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Reviews

New Members of the International Advisory Board

Reinhard Berner

Reinhard Berner is Professor of Pediatrics and since 2012 Chairman and Director of the Children's Hospital, University Medical Center Carl Gustav Carus at the Technische Universität Dresden, Germany. His research has focused on invasive infections in newborns and (young) children. His main interest is in the (molecular) epidemiology of group B and group A streptococci, viral respiratory tract infection, as well as the diagnosis of sepsis with novel technical approaches. He is experienced in various clinical aspects of pediatric infectious diseases, such as respiratory tract infections, antibiotic treatment strategies and antibiotic stewardship programs.

Reinhard Berner has been president of the German Society for Pediatric Infectious Diseases (DGPI), and is board member of the German Society for Pediatrics (DGKJ), and consultant board member of the Germany Society for Infectious Diseases (DGI). He is the main coordinator and chief editor of the "Handbook of Infectious Diseases in Children and Adolescents" (DGPI-Handbuch) edited by the German Society for Pediatric Infectious Diseases (DGPI).

Marcin Czyz

Marcin Czyz is a Consultant Spinal Neurosurgeon at The Royal Orthopaedic Hospital NHS Trust.

He undertook neurosurgical specialist training at Wroclaw University Hospital and Brighton and Sussex University Hospitals NHS Trust, graduating with the European Neurosurgical Diploma. He completed the Royal College of Surgeons and was accredited two-year fellowship in spine surgery in Nottingham. He was also granted the Neurooncology and Minimally Invasive Spinal Surgery Fellowship in New York.

The main focus of his work are spinal tumors (including intradural) and degenerative diseases of the cervical and lumbar spine.

Marcin Czyz has presented and published both nationally and internationally, completed with distinctions his Ph.D. thesis in biomechanics of spinal cord injuries in 2010 under the supervision of professor Włodzimierz Jarmundowicz. He is a member of the Advisory Editorial Board of *European Spine Journal*, the British Association of Spinal Surgeons, AO Spine, EuroSpine and Scientific Committee of NSpine.

Buddhadeb Dawn

Buddhadeb Dawn received his medical degree from Rajendra Medical College in 1993 and pursued his education in University of Missouri–Columbia (1994–1996) and University of Louisville (1996–2000). Since 2009 he is a Professor of Medicine with tenure at Division of Cardiology at University of Kansas Medical Center, Director of Division of Cardiovascular Diseases and Director of Cardiovascular Research Institute at University of Kansas Medical Center. In years 2009–2016 he was Vice Chair for Research, Department of Medicine at University of Kansas Medical Center. Since 2013 he is a Director of Midwest Stem Cell Therapy Center at University of Kansas.

He is a member of many associations and societies (American Heart Association, International Society for Heart Research, Association of Professors of Cardiology), a member of Editorial Board of various medical journals (*Circulation Research*, *American Journal of Physiology*: *Heart and Circulatory Physiology*, *Molecular Biology Reports*, *Clinical and Translational Medicine*) and a reviewer in many more (*J Am Coll Cardiol*, *Cardiovascular Research*, *Heart*, *Stem Cells*, *Stem Cells Dev*, *FEBS Lett*, *J Cell Mol Med*, *Gene*, *Int J Cardiol*, *Antioxidant Redox Signal*, *PLoS ONE*, *Regenerative Medicine*, *JAMA*, *Stem Cell Research*, *NEJM*, *Lancet*).

He won numerous Honors and Awards: National Talent Search Scholarship (1981–1993); College Scholarship, Calcutta Medical College (1984–1985); Junior Class Assistant, Pharmacology (1985–1986); 2nd Certificate of Honors, Pharmacology (1984–1985); Green-Armytage Silver Medal and Prize (1987–1988); 'Fellow of the year' Award, Division of Cardiology, University of Louisville (1996–1997 and 1997–1998); AHA Postdoctoral Fellowship Award (1997–1998 and 1998–1999); Chief Fellow, Division of Cardiology, University of Louisville (1999–2000); Outstanding Investigator Award, Dept. of Medicine, University of Louisville (2007); University Scholar, University of Louisville (2008); Maureen and Marvin Dunn Professorship, University of Kansas (2009); Southern Society for Clinical Investigation (2011); Castle Connelly US News Top Doctors (2011–); Fellow, International Academy of Cardiovascular Sciences (2014).

His research has been guided by the overarching philosophy that biomedical research should be translated to human therapy, whenever possible. In that regard, his areas of research interest have varied over the past two decades, and have included both basic molecular studies as well as clinical research.

Kishore Kumar Jella

Kishore Kumar Jella received BS in Microbiology, Chemistry and Computer Applications in 2003 and MS in Biochemistry in 2005 from the Osmania University, Hyderabad, India. In 2007 he received MS in Molecular Biology from University of Skövde in Sweden. In 2012 he received PhD in Radiation Induced Non-Targeted Signaling from Dublin Institute of Technology, Radiation and Environmental Science Center, Ireland.

In 2007–2008 he worked as Research Assistant at Charité University Hospital, Berlin, Germany. Since 2013 he has been a postdoctoral fellow at the Department of Radiation Oncology, Emory University School of Medicine.

He is a co-author of many manuscripts published in such journals as *Radiation Research*, *PLOS ONE* and *International Journal of Radiation Biology*. He received two research grants: Emory Winship ACS IRG (2014–2015) and Winship Invest$ Grant (2016–2018).

He is and used to be a member of many professional societies: Association of Radiation Research Society (2009–2012), Microscopical Society of Ireland (2010–2012), Irish Radiation Research Society (2011–2012), Radiation Research Society (2013–2017), European Radiation Research Society (2017) and won several awards: Early Career Investigator Travel Bursary Award (2011), MSI Travel Bursary Award (2011), Early Career Travel Bursary Award (2012), Outstanding Poster Award for the best poster in Cancer Biology & Oncology Sciences (2014), Scholars-in-Training Travel Award (2015).

Pavel Kopel

Pavel Kopel is an Associate Professor at Department of Chemistry and Biochemistry, Faculty of Agronomy, Mendel University in Brno and a Senior Researcher at Central European Institute of Technology, Brno, Czech Republic. He received his scientific degrees from the Department of Inorganic Chemistry, Faculty of Science, Palacky University in Olomouc. He is an author or co-author of over 100 ISI indexed papers with H-index 16 and a holder of two patents. His research is mainly focused on synthesis and characterization of organic and coordination compounds and nanomaterials for therapeutics, drug delivery, biosensors, separation, remediation and electrode modification. He participated on many national research projects and international projects including "Ultra-Fast Molecular Filovirus Diagnostics" funded by H2020 project scheme.

Tomasz Owczarek

He obtained his master's and doctoral degree from Wroclaw University of Environmental and Life Sciences (Wrocław, Poland). As a graduate student, mentored by Prof. Maciej Ugorski, his research was focused on mechanistic insights regarding the role of glycosyltransferases in breast cancer progression and metastasis. He was also involved in several collaborative studies aimed at defining novel markers of breast cancer malignancy and drug resistance in leukemia. During his postdoctoral research experience at Columbia University Medical Center (New York, USA) he has specialized in bladder cancer basic research. He is a recipient of American Urological Association Research Scholar Award founded by Urology Care Foundation for his studies on the cell of origin for bladder cancer.

It is his intention to dedicate his career to understanding the molecular mechanisms involved in the initiation and progression of bladder cancer in order to identify new therapeutic approaches for muscle invasive bladder cancer (MIBC) which

is the fatal form of the disease. He believes that the mysteries behind bladder cancer biology represent a major challenge for a molecular cancer biologist. Furthermore, he has a strong interest in the interplay between basic biological investigations and establishing their clinical relevance. It will be his great pleasure to serve as an advisory board member for *Advances in Clinical and Experimental Medicine*.

Ivan Rychlík

Ivan Rychlík is a Professor of Internal Medicine. He graduated (MD) in 1987, summa cum laude, from Charles University in Prague, Czech Republic. In 1994 he graduated in Internal Medicine and in 1996 in Nephrology. In 1998–1999 he received one-year Fellowship Award of ISN in Heidelberg (tutor Professor Eberhard Ritz). In 2001 he defended his PhD (Charles University, Prague). Since 1995, he is active in pre- and post-gradual teaching. In 2004 he habilitated as Associate Professor and in 2011 as Full Professor of Internal Medicine (Charles University, Prague). Since 1987 he has been employed by Charles University, Prague, and since 2005 he has worked as a Head of Dialysis Unit, Fresenius Medical Care in Faculty Hospital of the 3rd Faculty Medicine, Prague.

He has a long-lasting and practical experience in running different kinds of registries on national-wide level. He is a co-founder of the Czech Registry of Renal Biopsies (1994), co-founder and current Chairman of the Czech Registry of Dialysis patients (2004), and co-founder of the Czech Registry of CKD-4 patients (2010). He is an active member of several Societies: Czech Society of Nephrology (CSN), ERA-EDTA, ISN, ASN, EUVAS. In international field, apart from ERA-EDTA, he serves as a member of GO Committee of ISN for EEC. He is the Managing Editor of *Case Reports in Nephrology and Urology*, member of editorial boards of *Kidney Blood Pressure Research*, *Diabetes Research and Treatment* and several Czech medical journals (most important: *Current Opinion in Hypertension and Nephrology*). Furthermore, he is an active reviewer of several international journals (particularly *Nephrology Dialysis Transplantation*, *KBPR*, *Blood Purification*).

He is an author/co-author of 174 scientific articles (indexed in Medline), 9 monographs and 47 book chapters. He gave more than 300 presentations in international meetings. His current SCI is 1385 (WOS) and H-index is 17. He was a principal investigator in 3 scientific Czech grants (IGA Czech Ministry of Health) and co-investigator of 3 EU grants (QUEST, PREDICTIONS, PRIORITY), and other 8 Czech grants. Furthermore, he worked as principal or national investigator in several trials, mostly in the field of diabetic nephropathy (TREAT, SHARP, GFRF study, VIVALDI, etc.). His current main scientific interests are epidemiology of renal diseases, diabetic nephropathy and CVD in diabetic patients on dialysis treatment. He won the Year Award of Czech Medical Society for Best Monograph (1994), Award of ERA-EDTA for outstanding scientific presentation (Madrid, 1999), Jan Brod Award for the best publication in Nephrology (2000), Award of the CSN for the best publication (2005) and ISBP Best Abstract Award (Brijuni, 2008).

Anton Sculean

Anton Sculean is a Professor and Chairman of the Department of Periodontology and currently the Executive Director of the School of Dental Medicine, University of Bern, Switzerland. He graduated in 1990 at the Semmelweis University in Budapest, Hungary and has received his postgraduate training at the University of Münster, Germany and Royal Dental College of Aarhus, Denmark. He received his habilitation (PhD) at the Saarland University, Homburg, Germany. From 2004 to 2008 he was the Head of the Department of Periodontology and Program Director of the EFP accredited postgraduate program at the Radboud University, Nijmegen, the Netherlands. In December 2008, he was appointed Professor and Chairman of the Department of Periodontology of the University of Bern, Switzerland. Currently, he serves as Executive Chairman of the School of Dental Medicine, University of Bern. Professor Sculean has been a recipient of many research awards, among others the Anthony Rizzo Award of the Periodontal Research Group of the International Association for Dental Research (IADR), and the IADR/Straumann Award in Regenerative Periodontal Medicine. He received honorary doctorates (Dr h.c.) from the Semmelweis University in Budapest, Hungary and from the Victor Babes University of Medicine and Pharmacy in Timisoara, Romania.

He has authored more than 280 publications in peer-reviewed journals, has written 16 chapters in periodontal textbooks and has delivered more than 350 lectures at national and international meetings. He is the editor of the book *Periodontal Regenerative Therapy* published by Quintessence and a guest editor of the Periodontology 2000 Issue published in 2015 entitled *Wound Healing in Periodontology and Implantology*.

He serves on the editorial board of more than 12 dental journals, among others *Journal of Dental Research*, *Journal of Clinical Periodontology*, *Clinical Oral Implants Research*, *Journal of Periodontal Research and Clinical Advances in Periodontics* and is associate editor of *Quintessence International*, *Clinical Oral Investigations*, section editor of *BMC Oral Health* and editor in chief of *Oral Health and Preventive Dentistry*.

Professor Sculean served from 2009–2010 as president of the Periodontal Research Group of the IADR, was a president of the Swiss Society of Periodontology, and is a President Elect (for the period 2018–2019) of the European Federation of Periodontology (EFP). His research interests focus on periodontal wound healing, regenerative and plastic-esthetic periodontal therapy, use of antibiotics, antiseptics and novel approaches such as lasers and photodynamic therapy in the treatment of periodontal and peri-implant infections.

The effect of platelet-rich plasma in bone-tendon integration

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article

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Abstract

Background. The operative reconstruction of a torn or insufficient anterior cruciate ligament has become a routine surgical procedure in orthopedics. The long-term success of an anterior cruciate ligament reconstruction depends on the ability of the graft to heal adequately in a bone tunnel. Investigators studying reconstructions described healing within a tunnel as osseous ingrowth and incorporation. In particular, helping the healing using autologous material for the best integration process was a new idea that helped us to set up this study.

Objectives. The purpose of this study is to show the effect of platelet-rich plasma on bone-tendon healing.

Material and methods. Ten New Zealand rabbits were used. The study had 2 groups: (1) a study group including the right extremities of rabbits in which tendon-bone integration was strengthened by platelet-rich plasma and (2) a control group including the left extremities of rabbits in which tendon-bone integration was without platelet-rich plasma. On the 56th day postoperatively, the portion of the distal femur containing the tunnel was amputated following the euthanization process for histological evaluation.

Results. In the histological evaluation of the tendon-integrated bone segments with platelet-rich plasma, the integration of tendon in the bone was successful without any necrosis formation in most of the tissues. However, in the control group without platelet-rich plasma, the integration was distorted in many zones and some cystic morphologies were present.

Conclusions. The findings of this study showed that using platelet-rich plasma during tendon-to-bone implantation has positive effects histologically. In the literature, many studies are available that have investigated the effect of platelet-rich plasma on anterior cruciate surgery radiologically. However, the histological findings are more reliable than radiological findings because bone-tendon integration is a biological process.

Key words: platelet-rich plasma, anterior cruciate ligament, tendon, integration, revision surgery

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Lesions of the anterior cruciate ligament (ACL) represent one of the most common traumas in sporting practice and have shown a tendency to increase in the last few years.[1] Operative reconstruction of a torn or insufficient ACL has become a routine surgical procedure in orthopedics.[2] The most commonly used grafts for this procedure are autologous bone-patellar tendon-bone, hamstring, and quadriceps tendons. Biomechanical testing has shown that the initial strength of the graft materials is higher than that of the intact ACL.[3] Hence, the most fragile linkage between the 2 tissues is the femoral and tibial fixation points rather than the graft itself.[4] This finding has led to the commercial development of certain fixation materials. These materials are functional only before tissue healing occurs. Ultimately, the long-term success of an ACL reconstruction depends on the ability of the graft to heal adequately in a bone tunnel.

Researchers studying reconstructions have described healing within a tunnel as osseous ingrowth and incorporation in the surrounding tissue and then toward the tendon tissue.[5] Healing using autologous material for the best integration process was the idea that initiated this study.[6]

In 1990, Gibble and Ness introduced fibrin glue, alternatively referred to as fibrin sealant or fibrin gel, a biomaterial that was developed in response to the necessity for improved hemostatic agents with adhesive properties.[7]

Platelet-rich plasma (PRP) gel is an autologous modification of fibrin glue that has been described and used in various applications with apparent clinical success.[8] PRP obtained from autologous blood is used to deliver growth factors in high concentrations to the site of the bone defect or a region requiring augmentation.[9]

Late union of the graft and tunnel expansion due to repeated movement is a significant problem associated with primary and revision ACL surgery. It is believed that the PRP improves the integration between the bone and the graft.

Material and methods

A total of 10 New Zealand rabbits, weighing 3000––3500 g, were used. The study was comprised of 2 groups: A study group including the right extremities of the rabbits (10 right knees) in which bone-tendon integration was strengthened using PRP; and a control group including the left extremities of the rabbits (10 left knees) in which bone-tendon integration was performed without PRP. The Research Ethics Commission gave consent for the use of laboratory animals for the experiments (Marmara University, 24.03.2008–13.03.2008).

PRP preparation

The PRP was prepared using RegenKit THT Autologous Platelet-Rich Plasma (A-PRP) (RegenKit® THT, Stryker, Kalamazoo, USA), which is a PRP preparation with high platelet recovery and viability, physiological levels of leukocytes, and the entire plasma component of blood rich in growth factors.

All animals were anesthetized using 100 mg/kg intraperitoneal ketamine hydrochloride plus 3–5 mg/kg chlorpromazine. Eight milliliters of blood was collected from the marginal ear vein of the rabbits under strict aseptic conditions. The blood samples were transferred to a Regen THT tube that had thixotropic gel to separate the red blood cells from the whole blood. The samples were then centrifuged for 8 min at 3400 rpm, and PRP was extracted using the syringe.

Surgical procedure

After the blood samples were taken, a preoperative dose of intramuscular cefazolin sodium (0.1 mg/kg) was given for prophylaxis against infection. After the posterior side of the right ankle of the rabbits was shaved and before sterile covering, povidone iodine was used for prepping. Cutaneous and subcutaneous incisions were made over the Achilles tendon; 3 cm of the tendon was cut off from the insertion to the calcaneus and proximal musculotendinous part. The 2 ends of the graft were sutured with 2/0 vicryl using the Krackow technique, and the thicknesses of the tendons were measured.

The prepared tendons were covered in a wet gauge. Later the distal parts of both femurs were shaved and covered with sterile drapes. An approximately 2-cm incision was made anterior to the distal aspect of the right femur. After advancing through the skin and subcutaneous layers, the quadriceps was deviated laterally to reach the bone. After incising the periosteum, it was detached to the posterolateral aspect of the femur. Using a K-wire, a 1-cm deep tunnel was drilled with an appropriate-thickness (as the graft thickness) cannulated drill (Fig. 1). After the tunnel preparation, the tunnel was filled with PRP so that the graft was totally covered with PRP as it advanced into the tunnel. The graft was sutured to the posteromedial periosteum at the point where it exited the tunnel. The PRP was again applied inside the tunnel (Fig. 2). Later all the layers, skin, and subcutaneous tissue were closed in the appropriate order and a dressing was applied to the wounds. In the control group (left femurs), except for the PRP application, all other procedures were performed in the same manner.

Postoperative care

In the postoperative period, intramuscular cefazolin sodium 0.1 mg/kg was administered for 5 days for antibiotic prophylaxis. Every 3 days, the dressing was applied on both the incision areas. After 10 days, the sutures were removed. On the 56th day postoperatively, the portion of the distal femur containing the tunnel was amputated for histological evaluation.

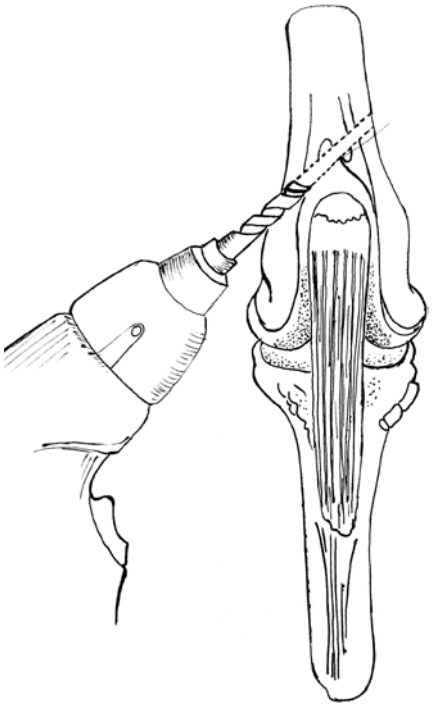

Fig. 1. Diagram showing the direction of the femoral tunnel

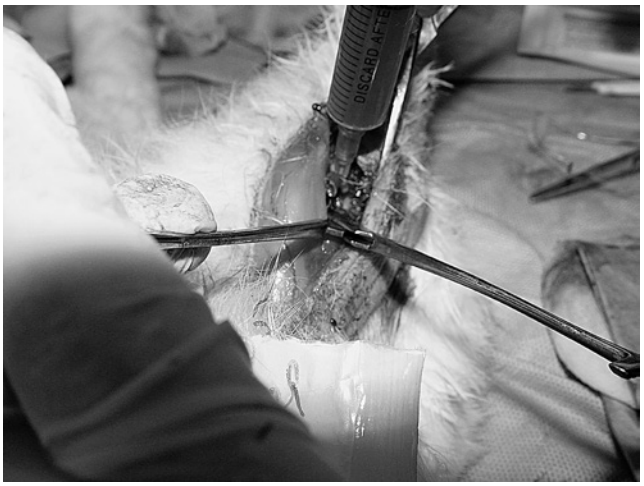

Fig. 2. PRP injected into the tunnel after the tendon was fixed

Preparation for microscopy

For light microscopy, the tissue samples were fixed in 10% buffered formalin for 48 h, dehydrated in ascending alcohol series, and embedded in paraffin wax. Approximately 7-µm-thick sections were stained with Masson's trichrome stain to demonstrate the general morphology.

Results

The histological evaluation revealed that the tendon-bone integration was successful in most of the tissues without any necrosis (Figs. 3Ra, Rb). Inflammatory cellularity in the integration zones was less in the study group compared to the control group. However, in the control group (Figs. 4La, Lb), the integration was distorted in many zones and some cystic morphologies were present (Table 1).

Table 1. Histologic scorin g (semi-quantitative histologic analysis)

Histological findings with Masson's Trichrome stain	Study group (10 right knees)	Control group (10 left knees)
Cellularity	+	+ + +
Morphologic disorganization in integration	–	+ + +
Edema	–	+ + +
Tendon layout	–	+ + +

Each parameter displays: (–) none, (+) mild, (+ +) moderate, or (+ + +) severe. Masson's Trichrome stain. ×100 and ×200 (insets).

Discussion

Autologous bone-patellar tendon-bone graft offers the strongest healing potential because it relies mainly on bone-to-bone integration between the graft bone plug and the tunnel wall.[10] However, it has some donor-site morbidity, which has led surgeons to search for alternative graft sources. Hamstring grafts are the most used alternative. These grafts have less donor-site morbidity, but the initial bone-tendon attachment is weaker than the bone-bone attachment, which limits rehabilitation and delays return to activity.[10] Hamstring grafts also have a slower rate of healing.[11,12] Hence, there was a need to look for ways to improve the bone-tendon healing, which was thought to be multifactorial, involving biomechanical and biological factors.[13] New fixation materials have been developed recently for more stable biomechanical bone-tendon fixation.[14] However, these materials are functional only before tissue healing occurs.

The long-term success of an ACL reconstruction depends on adequate graft healing into a bone tunnel.[15] For this reason, researchers have focused more on increasing integration using growth factors. Strategies to improve tendon-bone tunnel healing have focused on providing appropriate molecular signals and cell differentiation resulting in an effective healing response between tendon and bone.[16] Some studies have been demonstrated to improve bone ingrowth into a tendon graft placed in a bone tunnel with the use of osteoinductive cytokines.[16–19] Hyperbaric oxygen, low intensity pulsed ultrasound and extracorporeal shock waves could induce marked increases in vascularity, which improve new bone formation.[16,20,21] Osteoconductive materials may also play a role in improving tendon healing in a bone tunnel via enriched bone ingrowth.[16,18,20] Chen CH et al. evaluated the effect of a periosteum-enveloping tendon graft on tendon-bone healing in 2 different experimental models in rabbits and they concluded that

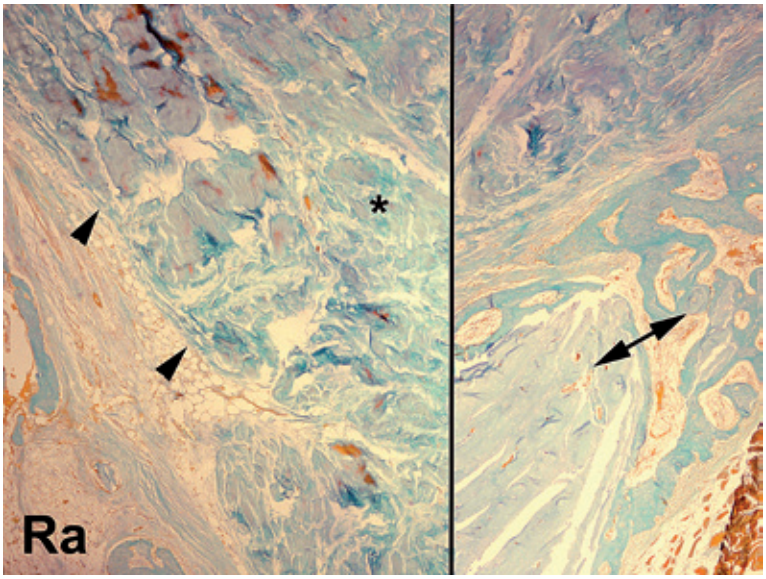

Fig. 3Ra. Integration zones of the tendon in bone (double-headed arrow); tendon integration points with connective tissue in periphery the (arrowheads); (*) indicates the tendon; Ra – right knee (study group) Fig. a

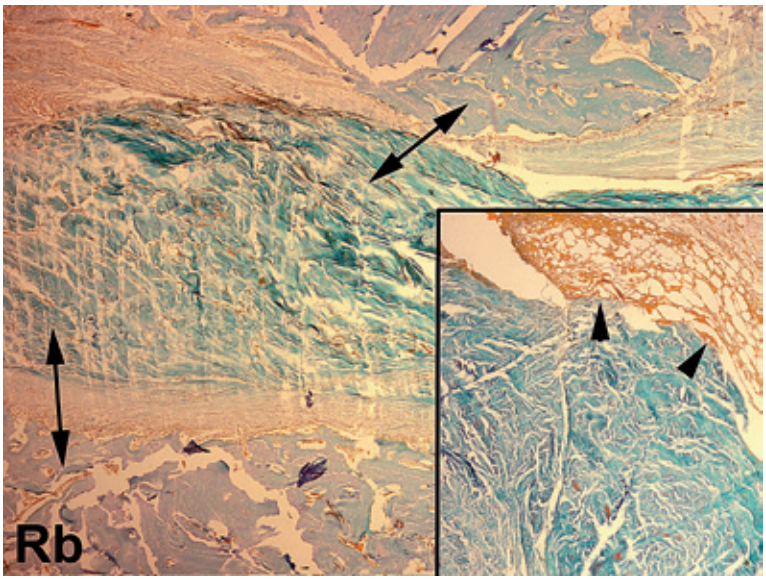

Fig. 3Rb. Integration of the tendon in bone tissue without any edema (double-headed arrows); the nidus of tendon integration (arrows); Rb – right knee (study group) Fig. b

a superior healing process and stronger healing strength could be achieved when the periosteum was sutured on the tendon graft inserted into a bone tunnel.[22,23] Chen CH et al. also evaluated the periosteal progenitor cells (PPC)-BMP-2 hydrogel for tendon-bone healing in 2 different experimental models and they concluded that PPC-BMP-2 is a powerful inducer of tendon-bone healing through the neoformation of fibrocartilage.[24,25]

PRP is rich in growth factors and proteins, such as fibrinogen.[26–28] The growth factors play an important role in the regulation of growth and development of a variety of tissues.[29] They accelerate the wound healing processes by increasing cellular proliferation, matrix formation, osteoid production, connective tissue healing, angiogenesis, and collagen synthesis.[30] PRP contains platelet-derived growth factor (PDGF), transforming growth factor beta 1 (TGF-β1), platelet-derived epidermal growth factor (PDEGF), platelet-derived angiogenesis factor (PDAF), insulin-like growth factor 1 (IGF-1), platelet factor 4 (PF-4), vascular endothelial growth factor (VEGF), fibroblast-derived growth factor (FGF), epidermal growth factor (EGF), and bioadhesive proteins such as fibrinogen, fibronectin, and vitronectin.[31,32]

There is no consensus regarding the use of growth factors in ACL surgery.[1,33,34] The structure of the ACL becomes tendon-like 9–12 months after surgery. Ventura et al. observed that the transformation from autologous quadrupled hamstring tendon graft (QHTG) to new ACL was faster in the PRP-treated group than in the controls. Hence, it was assumed that the growth factors, which were obtained using the Biomet-Merck Gravitational Platelet Separation (GPS) technique and applied to the femoral and tibial tunnels during the surgery, could accelerate the integration of the new ACL in the femoral and tibial tunnels. Computed tomography (CT) data confirmed this hypothesis.[1] In a study by Vogrin et al., PRP was applied to the femoral

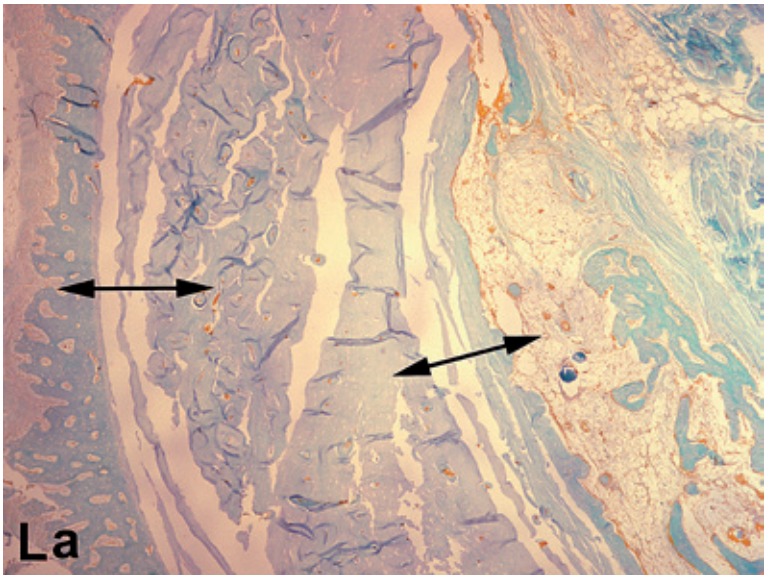

Fig. 4La. The edemateous interval between the bone and the tendon (double-headed arrows) where the integration is not proper; La – left knee (control group) Fig. a

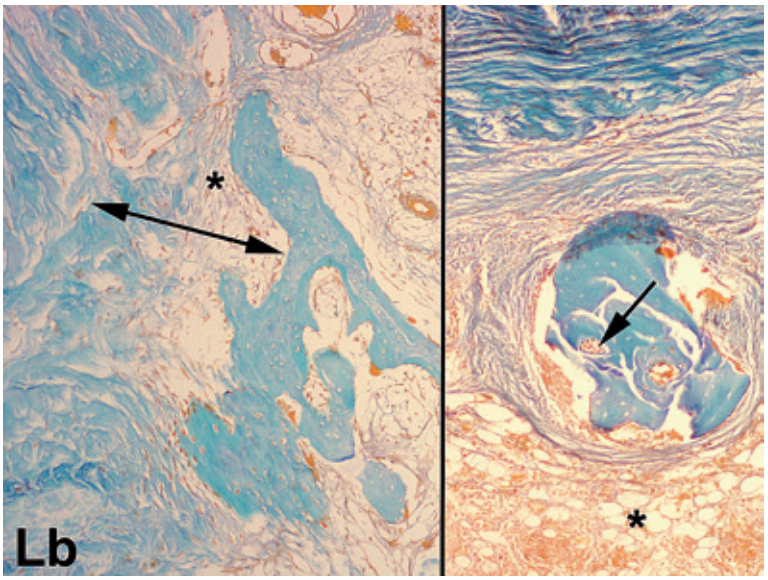

Fig. 4Lb. Edema is present in the integration zone (double-headed arrow) with prominent cellularity; (*) indicates cystic morphology in the tendon containing bone tissue (arrow); Lb – left knee (control group) Fig. b

and tibial tunnels as well as on the graft itself after autograft reposition.[35] Contrast-enhanced magnetic resonance imaging (MRI) studies were performed 4–6 and 10–12 weeks after the surgery. After 4–6 weeks, the PRP-treated group demonstrated a significantly higher level of vascularization in the osteoligamentous interface compared to the control group. It was concluded that locally applied PRP enhanced early revascularization of the graft in the osteoligamentous interface zone after ACL reconstruction. In a 2010 study by Silva et al., the patients were sequentially enrolled into 4 groups: Group A without PRP; group B with PRP in the femoral tunnels at the end of the surgery; group C with PRP in the femoral tunnels at the end of the surgery and intra-articularly 2 and 4 weeks after the surgery; and group D with PRP activated with thrombin in the femoral tunnels.[13] All patients underwent MRI of the knee 3 months after the surgery to evaluate the signal intensity of the fibrous interzone in the femoral tunnels. However, no difference was found among the groups when comparing the signal intensity of the fibrous interzone on MRI.

The results of the aforementioned studies were consistent with the radiological findings. However, histological findings are more reliable than CT and Magnetic Resonance Imaging (MRI) findings because bone-tendon integration is a biological phenomenon. The molecular findings are difficult to demonstrate with CT or MRI. In the present study, PRP was applied to the femoral tunnel after Achilles tendon graft reposition. After 8 weeks, bone-tendon integration was studied histologically. The bone-tendon integration in most of the tissues was successful without any necrosis in the study group. Also, inflammatory cellularity was less in the study group compared to the control group. However, the integration was distorted in many zones and some cystic morphologies were present in the control group. These findings indicate improved bone-tendon integration using PRP.

Browning et al. found that PRP contains a mixture of anabolic and catabolic mediators. Synoviocytes treated with platelet-rich plasma responded with secretion of matrix metalloproteinases, which might increase cartilage catabolism.[36] However, similar results were found in the study by Demirag et al. using alpha-2-macroglobulin.[11] Bilateral ACL reconstruction was performed with the use of the ipsilateral semitendinosus tendon in rabbits. Alpha-2-macroglobulin was injected into the knee joint in one limb, and the contralateral limb served as a control. Their results demonstrated that alpha-2-macroglobulin blockade of matrix metalloproteinases can enhance bone-tendon healing. Consequently, PRP and alpha-2-macroglobulin injection during the same procedure may increase the effect of PRP on bone-tendon integration.

Another issue regarding the growth factors in PRP is the varying effects of different growth factors of the same pool. Platelet sequestration results in a platelet concentrate that, upon activation, releases a cascade of growth factors contained in the alpha granules. The growth factors released from the platelets signal the local mesenchymal cells and epithelial cells to migrate, divide, and increase collagen and matrix formation. Each growth factors can be studied to distinguish their effects on bone-tendon integration by means of their biomechanical and histological properties. Some growth factors may have a negative influence on bone-tendon integration according to the study results, so extracting these growth factors may improve bone-tendon integration.

A limitation of this study was that it lacked biomechanical tests, extra-articular fixation, and tendon fixation techniques. Bone-tendon integration was also affected by intra-articular materials during healing, however this was fixed extra-articularly. The graft was sutured to the periosteum. It is not a strong fixation technique compared to other fixation techniques that are used in ACL surgeries. This might have affected the results of the present study results negatively.

PRP was used as a source of growth factors in this study and tendon graft integration was evaluated histologically. The bone-tendon integration was successful without any necrosis in the study group. The histological findings will be more reliable with radiological findings. However, the biomechanical properties of this integration still need to be explored.

References

1. Ventura A, Terzaghi C, Borgo E, Verdoia C, Gallazzi M, Failoni S. Use of growth factors in ACL surgery: Preliminary study. *J Orthop Traumatol*. 2005;6:76–79.
2. Ferrari JD, Bach BR. Isolated anterior cruciate ligament injury. In: Chapman MW, ed. *Chapman's Orthopaedic Surgery*. Philadelphia, PA: Lippincott, Williams and Wilkins; 2001:2347–2359.
3. Hamner DL, Brown CH Jr, Steiner ME, et al. Hamstring tendon grafts for reconstruction of the anterior cruciate ligament: Biomechanical evaluation of the use of multiple strands and tensioning techniques. *J Bone Joint Surg Am*. 1999;81:549–557.
4. Kurosaka M, Yoshiya S, Andrish JT. A biomechanical comparison of different surgical techniques of graft fixation in anterior cruciate ligament reconstruction. *Am J Sports Med*. 1987;15:225–229.
5. Martinek V, Latterman C, Usas A, et al. Enhancement of tendon-bone integration of anterior cruciate ligament grafts with bone morphogenetic protein-2 gene transfer: A histological and biomechanical study. *J Bone Joint Surg Am*. 2002;84:1123–1131.
6. Rodeo SA, Arnoczky SP, Torzilli PA, Hidaka C, Warren RF. Tendon-healing in a bone tunnel: A biomechanical and histological study in the dog. *J Bone Joint Surg Am*. 1993;75:1795–1803.
7. Gibble J, Ness P. Fibrin glue: The perfect operative sealant? *Transfusion*. 1990;30:741–747.
8. Hood AG, Hill AG, Reeder GD. Perioperative autologous sequestration. III: A new physiologic glue with wound healing properties. *Proc Am Acad Cardiovasc Perfusion*. 1993;14:126–130.
9. Marx RE, Carlson ER, Eichstaedt RM, Schimmele SR, Strauss JE, Georgeff KR. Platelet-rich plasma: Growth factor enhancement for bone grafts. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 1998; 85:638–646.
10. Silva A, Sampaio R. Anatomic ACL reconstruction: Does the platelet-rich plasma accelerate tendon healing? *Knee Surg Sports Traumatol Arthrosc*. 2009;17:676–682.
11. Demirag B, Sarisozen B, Ozer O, Kaplan T, Ozturk C. Enhancement of tendon-bone healing of anterior cruciate ligament grafts by blockage of matrix metalloproteinases. *J Bone Joint Surg Am*. 2005;87:2401–2410.
12. Kyung HS, Kim SY, Oh CW, Kim SJ. Tendon-to-bone tunnel healing in a rabbit model: The effect of periosteum augmentation at the tendon-to-bone interface. *Knee Surg Sports Traumatol Arthrosc*. 2003;11:9–15.
13. Silva A, Sampaio R, Pinto E. Femoral tunnel enlargement after anatomic ACL reconstruction: A biological problem? *Knee Surg Sports Traumatol Arthrosc*. 2010;18:1189–1194.
14. Weiler A, Hoffmann RF, Stähelin AC, Bail HJ, Siepe CJ, Südkamp NP. Hamstring tendon fixation using interference screws: A biomechanical study in calftibial bone. *Arthroscopy*. 1998;14:29–37.
15. Bachy M, Sherifi I, Zadegân F, Petrover D, Petite H, Hannouche D. Anterior cruciate ligament surgery in the rabbit. *J Orthop Surg Res*. 2013;19:8–27.
16. Chih-Hwa Chen. Strategies to enhance tendon graft-bone healing in Anterior Cruciate Ligament reconstruction. *Chang Gung Med J*. 2009;32:483–493.
17. Yamazaki S, Yasuda K, Tomita F, Tohyama H, Minami A. The effect of transforming growth factor-beta1 on intraosseous healing of flexor tendon autograft replacement of anterior cruciate ligament in dogs. *Arthroscopy*. 2005;21:1034–1041.
18. Gulotta LV, Kovacevic D, Ying L, Ehteshami JR, Montgomery S, Rodeo SA. Augmentation of tendon-to bone healing with a magnesium-based bone adhesive. *Am J Sports Med*. 2008;36, 1290–1297.
19. Rodeo SA, Suzuki K, Deng XH, Wozney J, Warren RF. Use of recombinant human bone morphogenetic protein-2 to enhance tendon healing in a bone tunnel. *Am J Sports Med*. 1999;27:476–488.
20. Walsh WR, Stephens P, Vizesi F, Bruce W, Huckle J, Yu Y. Effects of low-intensity pulsed ultrasound on tendon bone healing in an intra-articular sheep knee model. *Arthroscopy*. 2007;23:197–204.
21. Wang CJ, Wang FS, Yang KD, Weng LH, Sun YC, Yang YJ. The effect of shock wave treatment at the tendonbone interface: A histomorphological and biomechanical study in rabbits. *J Orthop Res*. 2005;23:274–280.
22. Chen CH, Chen WJ, Shih CH. Enveloping of periosteum on the tendon graft to enhance tendon-bone healing in a bone tunnel: A biomechanical and histological studies in rabbits. *Arthroscopy*. 2003;19:290–296.
23. Chen CH, Chen LH, Chen WJ, et al. Periosteum-enveloping of the tendon to enhance tendon-bone healing in the bone tunnel: Histologic studies in three experimental rabbit models. *J Orthop Surg Taiwan*. 2003;20:21–29.
24. Chen CH, Liu HW, Tsai CL, Yu CM, Lin IH, Hsiue GH. Photoencapsulation of bone morphogenetic protein-2 and Periosteal progenitor cells improve tendon graft healing in a bone tunnel. *Am J Sports Med*. 2008;36:461–473.

25. Chen CH, Liu HW, Hsiue GH, Tsai CL, Yu CM, Lin IH. Photoencapsulation of bone morphogenetic protein-2 and Periosteal progenitor cells enhance tendon-bone healing in anterior cruciate ligament reconstruction. *Trans Orthop Res Soc*. 2008;33:255.
26. Cieślik-Bielecka A, Bielecki T, Gaździk TS, Arendt J, Król W, Szczepański T. Autologous platelets and leukocytes can improve healing of infected high-energy soft tissue injury. *Transfus Apher Sci*. 2009;41:9–12.
27. Clark RA. Fibrin and Wound Healing. *Ann N Y Acad Sci*. 2001;936:355–367.
28. Whitman DH, Berry RL, Green DM. Platelet gel: An autologous alternative to fibrin glue with applications in oral and maxillofacial surgery. *J Oral Maxillofac Surg*. 1997;55:1294–1299.
29. Mishra A, Woodall J Jr, Vieira A. Treatment of tendon and muscle using platelet-rich plasma. *Clin Sports Med*. 2009;28:113–125.
30. Rick GS, Craig JG, Mark SC. Platelet-rich plasma: Properties and clinical applications. *J Lancaster Gen Hosp*. 2007;2:73–77.
31. Ganio C, Tenewitz FE, Wilson RC, Maules BG. The treatment of chronic nonhealing wounds using autologous platelet-derived growth factors. *J Foot Ankle Surg*. 1993;32:263–268.
32. Paoloni J, De Vos RJ, Hamilton B, Murrell GA, Orchard J. Platelet-rich plasma treatment for ligament and tendon injuries. *Clin J Sport Med*. 2011;21:37–45.
33. Schmidt CC, Georgescu HI, Kwoh CK, et al. Effect of growth factors on the proliferation of fibroblasts from the medial collateral and anterior cruciate ligaments. *J Orthop Res*. 1995;13:184–190.
34. Yoshida M, Fuji K. Differences in cellular properties and responses to growth factors between human ACL and MCL cells. *J Orthop Sci*. 1999;4:293–298.
35. Vogrin M, Rupreht M, Dinevski D, et al. Effects of a platelet gel on early graft revascularization after anterior cruciate ligament reconstruction: A prospective, randomized, double-blind, clinical trial. *Eur Surg Res*. 2010;45:77–85.
36. Browning SR, Weiser AM, Woolf N, et al. Platelet-rich plasma increases matrix metalloproteinases in cultures of human synovial fibroblasts. *J Bone Joint Surg Am*. 2012;5:1721–1727.

IFN-γ induces senescence-like characteristics in mouse bone marrow mesenchymal stem cells

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Conflict of interest
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Abstract

Background. Mesenchymal stem cells (MSC) are considered promising in tissue repair and regeneration medicine due to their proliferation and differentiation ability. Many properties of MSC are affected by cytokines, and IFN-γ has been shown to regulate MSC in many aspects. Senescence affects the proliferation, differentiation and cytokine secretion of MSC.

Objectives. To investigate the effects of IFN-γ on the senescence-associated properties of MSC.

Material and methods. The MSC used in our study were isolated from the bone marrow (BM) of mice. Cell vitalities were measured by CCK8. The phenotypes and ROS of mBM-MSC were analyzed by flow cytometry. Cellular senescence was detected using SA-β-gal stains. IL-6 and CXCL1 secretions were measured by ELISA.

Results. mBM-MSC can differentiated into osteocytes and adipocytes. They expressed CD29, CD106, and Sca-1, and did not express CD31, CD45 or FLK1. Our study showed that the cell vitalities of mBM-MSC were significantly reduced after IFN-γ treatment for 5 days, and the cell numbers were obviously lower after IFN-γ treatment for 5, 10 or 15 days. The IFN-γ group increased SA-β-gal-positive cells and reactive oxygen species (ROS) significantly after 15 days of IFN-γ treatment. Moreover, IL-6 and CXCL1 secretions were up-regulated by IFN-γ.

Conclusions. Our study shows IFN-γ can induce senescence-like characteristics in mBM-MSC, suggesting a novel target for anti-aging therapy.

Key words: IFN-γ, senescence, mesenchymal stem cells

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Mesenchymal stem cells (MSC) have been isolated from many kinds of tissues, and MSC derived from bone marrow have been studied the most. MSC proliferate in culture flasks quickly in vitro and differentiate into cells of the mesoderm, such as osteoblasts, adipocytes, chondrocytes, tendon and muscle cells under certain conditions.[1] Further research has also shown that MSC can differentiate into cells of the ectoderm and endoderm.[2] Thus, MSC is supposed to be promising in tissue repair and regeneration medicine.

MSC undergoes genomic mutation after long term in vitro culture, and becomes senescent.[3] The senescence of MSC in vitro has been shown to be a continuous process, and senescence progress starts in the early passages.[4] Additionally, MSC can be induced to premature senescence. Oxidative stress was one of the major inducers of premature senescence of MSC.[5] Like other cells, the proliferations of MSC were reduced by cellular senescence, and senescent MSC were stained by SA-β-gal.[6] The osteogenic and adipogenic differentiation potential of MSC were reduced by cellular senescence.[7] Moreover, the cytokine secretion of MSC was affected.[8] Thus, senescence affects many characteristics of MSC, and influences their in vivo function.

MSC are able to regulate the immune response and change the cytokine secretion of immune cells.[9] IFN-γ is one of the major cytokines for Th1 response, which is critical for cellular immunity. MSC can suppress IFN-γ production of $CD4^+$, $CD8^+$ and NK cells, and IFN-γ changes many properties of MSC.[9] IFN-γ induces MSC-expressed IDO1, which inhibits the proliferation of immune cells, and MSC itself.[10,11] IFN-γ also inhibits the osteogenic and adipogenic differentiation potential of human and mouse MSC.[11] CD106 and CD54 expressions on MSC could be up-regulated by IFN-γ, and they are important for the hematopoietic support and immune modulation abilities of MSC.[12] Moreover, IFN-γ is also an inducer of the chemokine secretion of MSC.[13] IFN-γ has been shown to induce senescence of many kinds of cells, including cancer cells, melanocytes and endothelial cells.[14–16] IFN-γ reduces proliferation of these cells while inducing ROS and DNA damage signaling. However, research on the effects of IFN-γ on stem cell senescence is limited.

In our study, we studied the effect of IFN-γ on mBM-MSC senescence-associated properties. We discovered that IFN-γ could suppress the proliferations of mBM-MSC and induce senescence-like characteristics of mBM-MSC. Additionally, IFN-γ regulated the senescence-associated cytokine IL-6 and CXCL1 production of mBM-MSC.

Material and methods

Generation of mouse bone marrow mesenchymal stem cells

Bone marrow cells were collected from 6–10 week old C57BL/6 mice by flushing femurs and tibias with 2 mL needles. The cells were seeded in a flask at a density of $10^6/cm^2$. The basic culture medium for the isolation of MSC was the MesenCult™ Proliferation Kit (Stemcell Technologies, Vancouver, Canada) or Dulbecco's modified Eagle's medium containing 10% FBS. Three days later, non-adherent cells were removed from the culture. The cells were cultured at 37°C in an atmosphere maintaining 5% CO_2 and passed at 80% confluence. Cells of passage 7 to passage 12 were used. The experimental procedures used in this study had been approved by the ethics committee within Zhejiang Hospital.

Flow cytometric analysis

The phenotypes of mBM-MSC were analyzed using the following antibodies: PE-Cy7-conjugated-CD31; PE-conjugated-CD29, CD45, CD106; APC-conjugated-FLK1 and Sca-1. Non-specific isotype-matched antibodies served as controls. All of the antibodies were purchased from eBioscience (San Diego, USA). Cells were analyzed using flow cytometry in a Beckman Coulter FC 500, and the data was analyzed using the FlowJo software (FlowJo LLC, Ashland, USA).

Osteogenic and adipogenic differentiation

mBM-MSC were plated in 24-well plates at a density of 3000 cells/cm^2. The medium was changed with specific induction medium 24 h later. For osteogenic and adipogenic differentiation induction, kits were purchased from Cyagen Biosciences Inc. (Santa Clara, USA). After 3 weeks of induction, the cells were stained using Alizarin red S or oil red O solution.

Proliferation assay

For the cell vitality assay, mBM-MSC were seeded 10^3 per well in 0.1 mL of DMEM with 10% FBS in 6-well plates. In the IFN-γ stimulated group, IFN-γ (1 ng/mL or 10 ng/mL) was added. The cells were analyzed using a Cell Counting Kit-8 (Beyotime Biotechnology, Shanghai, China). For cumulative population doublings (CPD) assay, mBM-MSC were seeded 5×10^4 per well in 2 mL of DMEM with 10% FBS in 6-well plates, and the medium changed after adherence to the flask. In the IFN-γ stimulated group, IFN-γ (10 ng/mL) was added. Cells were detached in the 5^{th}, 10^{th} and 15^{th} days. The cells were counted and CPD were calculated.

Senescence-associated beta-galactosidase (SA-β-gal) staining

mBM-MSC stimulated by IFN-γ (10 ng/mL) for 15 days were studied. The control group was mBM-MSC cultured without IFN-γ for 15 days. The culture mediums were discarded, and cells were washed with phosphate buffered saline. SA-β-gal staining was performed using a SA-β-gal staining kit (Genmed Scientifics Inc., Plymouth, USA) following the supplier's instructions. The percentages of SA-β-gal positive cells out of the total number of cells were counted.

Reactive oxygen species detection

mBM-MSC stimulated by IFN-γ (10 ng/mL) for 15 days were studied. Culture mediums were discarded, and the cells were washed with phosphate-buffered saline. The cells were incubated with 10 μM H2DCFDA (Sigma-Aldrich, St. Louis, USA) for 30 min and ROS were detected by flow cytometry.

ELISA

mBM-MSC stimulated by IFN-γ (10 ng/mL) for 15 days were isolated and seeded 3 × 10^4 per well in 0.5 mL of DMEM with 10% FBS in 24-well plates. The IFN-γ group was treated with 10 ng/mL. Cell-free supernatants were collected 24 h later and kept in a refrigerator at –80°C. IL-6 and CXCL1 ELISA kits were purchased from eBioscience (San Diego, USA), which were used following the supplier's instructions.

Statistical analysis

The data was analyzed for statistical significance using GraphPad Prism software (San Diego, USA). The data was presented as mean ± SEM. Student's unpaired t-test and ANOVA with Bonferroni post-hoc test were used to determine significance. $P < 0.05$ was considered to be statistically significant.

Results

Isolation of mouse bone marrow mesenchymal stem cells

After seeding, the bone marrow cells adhered to the flask, and proliferated quickly in the culture. Unlike in humans, several kinds of cells with different appearances could proliferate in the first few passages. Flow cytometry analysis showed that there were large amounts of $CD45^+$ cells.The percentage of $CD45^+$ cells became very

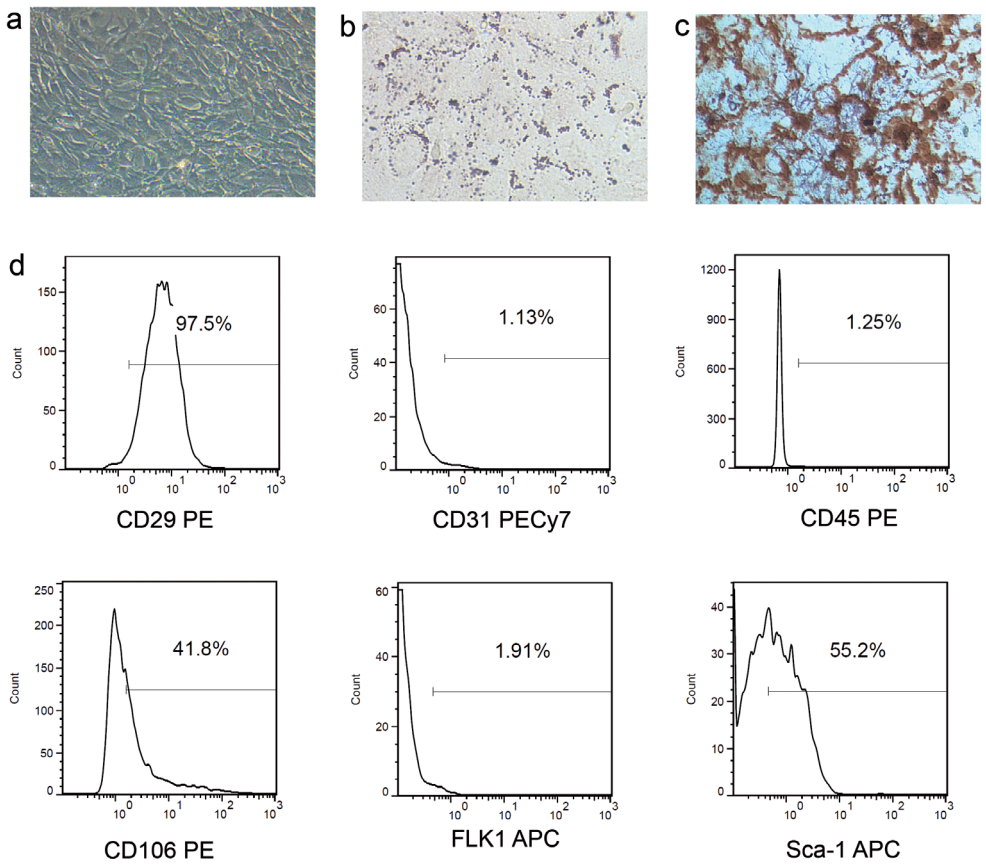

Fig. 1. Isolation of mBM-MSC. Cells of passage 7 to passage 12 were used. mBM-MSC were tested from 3 C57BL/6 mice and shown. Images for morphology and differentiation of mBM-MSC were taken by LEICA DMIL LED microscope

Fig. 1a. Morphology of mBM-MSC

Fig. 1b. Adipogenic differentiation potential of mBM-MSC. Cells were stained using oil red O

Fig. 1c. Osteogenic differentiation potential of mBM-MSC. Cells were stained using Alizarin red S

Fig. 1d. Phenotypes of mBM-MSC. Cells were analyzed by flow cytometry in a Beckman Coulter FC 500 cytometer, and the data was analyzed using the FlowJo software

low after passage 6 and the cells become uniform (Fig. 1a). Thus, the cells of passage 7 to passage 12 were used for the experiments. We tested their differentiation abilities and phenotypes. They differentiated into osteocytes and adipocytes under certain differentiation-inducing conditions (Figs. 1b, c). They expressed CD29, CD106, and Sca-1, while not expressing CD31, CD45 and FLK1 (Fig. 1d).

IFN-γ inhibit proliferation of mBM-MSC

We tested the effects of IFN-γ on mBM-MSC proliferation. One ng/mL and 10 ng/mL IFN-γ were used to stimulate mBM-MSC. After 120 h, CCK8 were used to test cell vitalities. The cell vitalities were significantly reduced by either concentration of IFN-γ (Fig. 2a, $p < 0.001$), and the OD in the 10 ng/mL IFN-γ group were lower than the OD in the 1 ng/mL IFN-γ group. Because of that, we used 10 ng/mL IFN-γ to treat mBM-MSC for 15 days and tested its effect on mBM-MSC proliferation. In the IFN-γ group, proliferation of mBM-MSC was reduced significantly after 5, 10 or 15 days' treatment (Fig. 2b, $p < 0.05$ after 5 days, $p < 0.001$ after 10 and 15 days).

IFN-γ induce mBM-MSC SA-β-gal and ROS

As the proliferation potentials of mBM-MSC were reduced by IFN-γ, we supposed that IFN-γ might be an inducer of mBM-MSC senescence. Therefore, we stained the SA-β-gal on the mBM-MSC. The percentages of SA-β-gal positive cells in the IFN-γ group were almost 3 times that of the control group (Figs. 3a and b, $p < 0.01$). ROS were suggested to be important for premature senescence. As expected, ROS were up-regulated in mBM-MSC after 15 days of IFN-γ treatment (Fig. 4).

IFN-γ up-regulated IL-6 and CXCL1 production of mBM-MSC

We tested the IL-6 and CXCL1 production after 15 days of IFN-γ treatment. IL-6 secretions were significantly up-regulated after 15 days of IFN-γ treatment (Fig. 5a, 5.21 ± 0.27 pg/mL in the control group vs 49.97 ± 2.86 pg/mL in the IFN-γ group, $p < 0.001$). Additionally, the CXCL1 secretions were up-regulated significantly (Fig. 5b, 17.61 ± 1.00 pg/mL in the control group vs 40.92 ± 2.64 pg/mL in the IFN-γ group, $p < 0.01$).

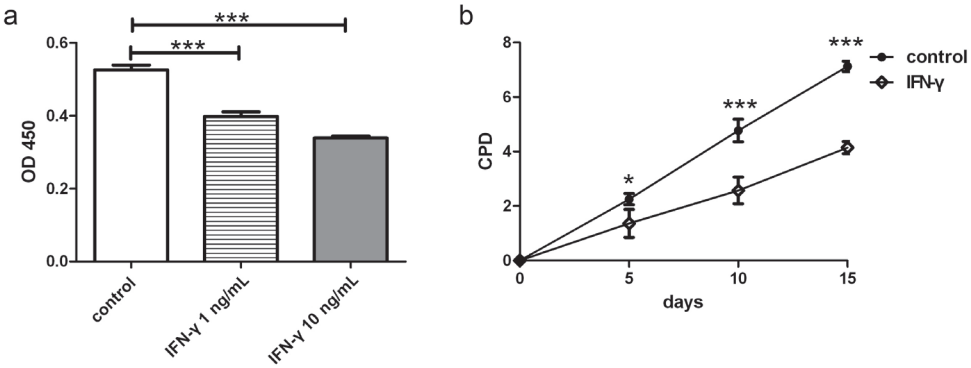

Fig. 2. IFN-γ inhibited proliferation of mBM-MSC

Fig. 2a. mBM-MSC were cultured with IFN-γ (1 ng/mL or 10 ng/mL) for 120 h, and cell vitalities were detected by CCK8. The data represents a single experiment, each performed in triplicate (*** $p < 0.001$)

Fig. 2b. mBM-MSC were cultured with IFN-γ (10 ng/mL) for 15 days. The cells were processed and counted. CPD were calculated. The data represents a single experiment, each performed in triplicate (* $p < 0.05$, *** $p < 0.001$)

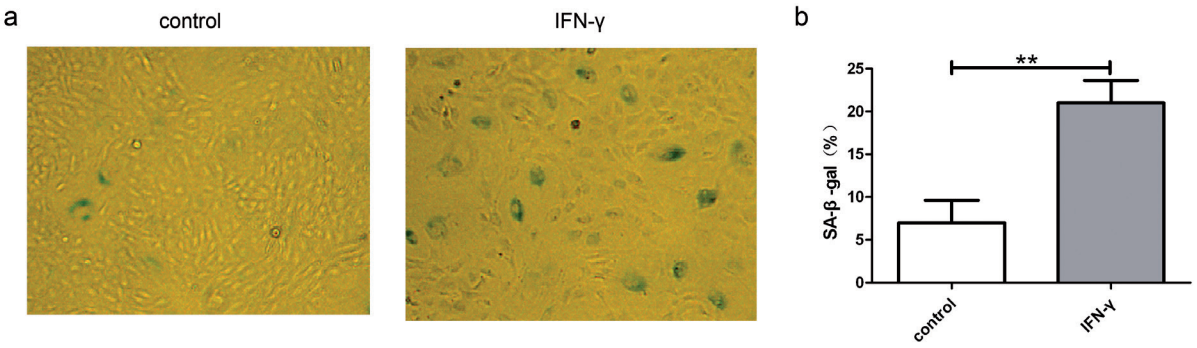

Fig. 3. IFN-γ induced SA-β-gal of mBM-MSC. mBM-MSC were cultured with IFN-γ (10 ng/mL) for 15 days

Fig. 2a. Cells of none-confluent state were washed with PBS, fixed with 4% formaldehyde and stained in a staining solution containing 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactoside for 16 h. Images were taken by LEICA DMIL LED microscope

Fig. 2b. Percentages of SA-β-gal positive cells out of the total number of cells were counted. The data represents a single experiment, each performed in triplicate (** $p < 0.01$)

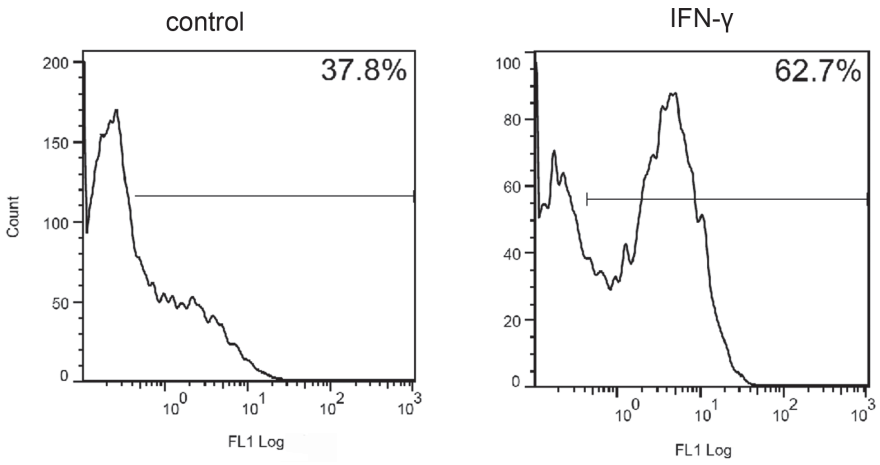

Fig. 4. IFN-γ induced ROS of mBM-MSC. mBM-MSC were cultured with IFN-γ (10 ng/mL) for 15 days. ROS of mBM-MSC from the untreated group and IFN-γ group were detected by flow cytometry. The data represents a single experiment, each performed in triplicate

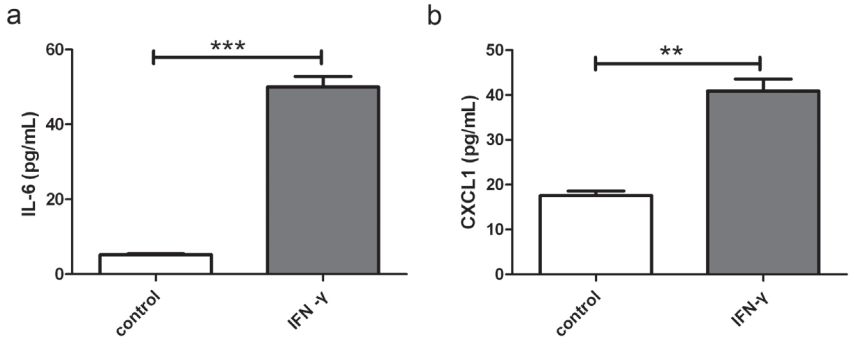

Fig. 5. IFN-γ up-regulated IL-6 and CXCL1 secretion of BM-MSC. mBM-MSC were cultured with IFN-γ (10 ng/mL) for 15 days. The cells were isolated and counted. 3×10^4 cells were seeded into a well of 6-well plates. The IFN-γ group was treated with IFN-γ (10 ng/mL). The culture medium was collected 24 h later, and concentrations of IL-6 (a) and CXCL1 (b) were tested by ELISA. The data represents a single experiment, each performed in triplicate (** $p < 0.01$, *** $p < 0.001$)

Discussion

Mesenchymal stem cells are populations of stem cells possessing great proliferation potential, while senescence limits its proliferate and function. Many studies have been done on replicative senescence of MSC before our research, and our data shows that the Th1 cytokine IFN-γ accelerates senescence-like characteristics in mBM-MSC.

Consistent with other research, IFN-γ reduced the proliferations of mBM-MSC in our study. A long-term in vitro culture with IFN-γ induced SA-β-gal expression of MSC, suggesting the role of IFN-γ on the dysfunctions of many organs in aging. Studies have shown that MSC could regulate the immune response, especially inhibiting the Th1 response.[10,17,18] It has been suggested that the IFN-γ produced by T/B/NK cells could activate the immune modulation effects of MSC, and MSC inhibits the proliferation and IFN-γ production of these cells.[10,18] Our data suggests high concentrations of IFN-γ could be an inducer of MSC senescence, and inflammatory cytokines could cause damage to MSC. It has been suggested that IFN-γ is an inducer of reactive oxygen species in endothelial cells, hepatocytes and melanocytes.[15,16,19] In our study, reactive oxygen species rose after 15 days of IFN-γ treatment. ROS are thought to be one of the major factors that cause cellular DNA damage, and subsequent senescence.[20] Thus, we suppose that IFN-γ induced ROS could be the cause of senescence of mBM-MSC.

Cellular senescence induces many cytokines, which was suggested to be the senescence-associated secretory phenotype (SASP).[21] SASP is controlled by NF-κB, and could exacerbate cellular senescence.[22] IL-6 and CXCL1 were important cytokines in the SASP of mice.[23] The up-regulation of IL-6 and CXCL1 by IFN-γ were consistent with the inducing senescence of MSC by IFN-γ. IL-6 is an important cytokine that regulates immune cells like B cells, Th17 cells and monocytes, so the effects of increasing IL-6 production of MSC might affect the immune responses.[24] Additionally, CXCL1 is related to immune response and angiogenesis.[25,26] Thus, 15 days of IFN-γ treatment might change the immune modulation and supportive effect of mBM-MSC. Other cytokines might also be influenced, and further studies are needed.

In summary, we have discovered that IFN-γ could induce senescence-like characteristics of mBM-MSC, suggesting a novel target for anti-aging therapy. Further studies will focus on the change of differentiation, immune modulation and supportive potential of MSC, and the mechanisms of IFN-γ induced MSC senescence.

References

1. Caplan AI. Mesenchymal stem cells. *J Orthop Res*. 1991;9:641–650.
2. Jiang Y, Jahagirdar BN, Reinhardt RL, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature*. 2002;418:41–49.

3. Wang Y, Zhang Z, Chi Y, et al. Long-term cultured mesenchymal stem cells frequently develop genomic mutations but do not undergo malignant transformation. *Cell Death Dis*. 2013;4:e950.
4. Wagner W, Horn P, Castoldi M, et al. Replicative senescence of mesenchymal stem cells: A continuous and organized process. *PLoS One*. 2008;3:e2213.
5. Choo KB, Tai L, Hymavathee KS, et al. Oxidative stress-induced premature senescence in Wharton's jelly-derived mesenchymal stem cells. *Int J Med Sci*. 2014;11:1201–1207.
6. Estrada JC, Torres Y, Benguria A, et al. Human mesenchymal stem cell-replicative senescence and oxidative stress are closely linked to aneuploidy. *Cell Death Dis*. 2013;4:e691.
7. Bonab MM, Alimoghaddam K, Talebian F, Ghaffari SH, Ghavamzadeh A, Nikbin B. Aging of mesenchymal stem cell in vitro. *BMC Cell Biol*. 2006;7:14.
8. Kasper G, Mao L, Geissler S, et al. Insights into mesenchymal stem cell aging: Involvement of antioxidant defense and actin cytoskeleton. *Stem Cells*. 2009;27:1288–1297.
9. Nauta AJ, Fibbe WE: Immunomodulatory properties of mesenchymal stromal cells. *Blood*. 2007;110:3499–3506.
10. Krampera M, Cosmi L, Angeli R, et al. Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. *Stem Cells*. 2006;24:386–398.
11. Croitoru-Lamoury J, Lamoury FM, Caristo M, et al. Interferon-gamma regulates the proliferation and differentiation of mesenchymal stem cells via activation of indoleamine 2,3 dioxygenase (ido). *PLoS One*. 2011;6:e14698.
12. Ren G, Zhao X, Zhang L, et al. Inflammatory cytokine-induced intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in mesenchymal stem cells are critical for immunosuppression. *J Immunol*. 2010;184:2321–2328.
13. Ren G, Zhang L, Zhao X, et al. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. *Cell Stem Cell*. 2008;2:141–150.
14. Braumuller H, Wieder T, Brenner E, et al. T-helper-1-cell cytokines drive cancer into senescence. *Nature*. 2013;494:361–365.
15. Wang S, Zhou M, Lin F, et al. Interferon-gamma induces senescence in normal human melanocytes. *PLoS One*. 2014;9:e93232.
16. Kim KS, Kang KW, Seu YB, Baek SH, Kim JR. Interferon-gamma induces cellular senescence through p53-dependent DNA damage signaling in human endothelial cells. *Mech Ageing Dev*. 2009;130:179–188.
17. Chen K, Wang D, Du WT, et al. Human umbilical cord mesenchymal stem cells huc-mscs exert immunosuppressive activities through a pge2-dependent mechanism. *Clin Immunol*. 2010;135:448–458.
18. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood*. 2005;105:1815–1822.
19. Watanabe Y, Suzuki O, Haruyama T, Akaike T. Interferon-gamma induces reactive oxygen species and endoplasmic reticulum stress at the hepatic apoptosis. *J Cell Biochem*. 2003;89:244–253.
20. Weyemi U, Lagente-Chevallier O, Boufraqech M, et al. Ros-generating nadph oxidase nox4 is a critical mediator in oncogenic h-ras-induced DNA damage and subsequent senescence. *Oncogene*. 2012;31:1117–1129.
21. Coppe JP, Patil CK, Rodier F, et al. Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic ras and the p53 tumor suppressor. *PLoS Biol*. 2008;6:2853–2868.
22. Chien Y, Scuoppo C, Wang X, et al. Control of the senescence-associated secretory phenotype by nf-kappab promotes senescence and enhances chemosensitivity. *Genes Dev*. 2011;25:2125–2136.
23. Coppe JP, Patil CK, Rodier F, et al. A human-like senescence-associated secretory phenotype is conserved in mouse cells dependent on physiological oxygen. *PLoS One*. 2010;5:e9188.
24. Chomarat P, Banchereau J, Davoust J, Palucka AK. IL-6 switches the differentiation of monocytes from dendritic cells to macrophages. *Nat Immunol*. 2000;1:510–514.
25. Ritzman AM, Hughes-Hanks JM, Blaho VA, Wax LE, Mitchell WJ, Brown CR. The chemokine receptor cxcr2 ligand kc (cxcl1) mediates neutrophil recruitment and is critical for development of experimental lyme arthritis and carditis. *Infect Immun*. 2010;78:4593–4600.
26. Dhawan P, Richmond A. Role of cxcl1 in tumorigenesis of melanoma. *J Leukoc Biol*. 2002;72:9–18.

In vivo effects of curcumin and deferoxamine in experimental endometriosis

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D – writing the article; E – critical revision of the article; F – final approval of article

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Abstract

Background. Endometriosis is one of the most common chronic gynecological diseases.

Objectives. The aim of the study was to examine the effects of curcumin and/or deferoxamine on cell proliferation in a rat model of endometriosis.

Material and methods. Thirty female 12-week-old albino Wistar rats, weighing 200–250 g, were used in this study. All the rats underwent ovariectomy and 0.1-mg β-estradiol 17-valerate pellets were placed intraperitoneally. An experimental model of endometriosis was created in all the animals. To create the experimental model, an approximately 1-cm long section of the uterus was taken, primarily from the right horn of the uterus. Autologous fragments were then placed between the peritoneum and muscle. The animals were divided into 3 groups: Group A, treated only with the vehicle used for curcumin and deferoxamine; group B, treated with curcumin (100 mg/kg body weight); and group C, treated with deferoxamine + curcumin (100 mg/kg body weight). After biopsy samples were obtained, the sections were stained with hematoxylin and eosin. Immunostaining for cytokeratin-7 and proliferating cell nuclear antigen (PCNA) was performed. Blood iron levels were measured using a Perkin Elmer AAnalyst 800 Atomic Absorption Spectrophotometer.

Results. The endometrial implant size increased in Group A, but treatment with curcumin ($p = 0.01$) and deferoxamine + curcumin ($p = 0.007$) reduced the implant size. In ectopic endometrial epithelial cells, there were significant decreases in PCNA immunoreactivity between groups A and B ($p = 0.044$) and between groups A and C ($p = 0.033$).

Conclusions. Treatment with curcumin alone and/or in combination with deferoxamine contributed to a reduction in implant size and cell proliferation in a rat endometriosis model. Iron-chelating agents may act in the same manner when used in women with endometriosis; however, further studies from different perspectives are still needed.

Key words: curcumin, endometriosis, deferoxamine, PCNA

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Endometriosis is one of the most common chronic gynecological diseases; it causes infertility, pelvic pain, dyspareunia and dysmenorrhea. The pathogenesis of this disorder remains unknown.[1] Histologic and cellular changes observed in eutopic and ectopic endometrial tissues from experimentally induced endometriosis are almost identical to those observed in women with endometriosis.[2–4] Iron levels are reportedly increased in women with endometriosis and in research animals with experimentally induced endometriosis.[5–7] Iron plays an important role in the progression of endometriosis because it affects cell proliferation.[6,7]

Deferoxamine is an iron-chelating agent that is used to remove iron from the blood. Treatment with deferoxamine or with curcumin has produced significant results for some patients with endometriosis, pancreatic cancer and β-thalassemia.[6,8–10]

Curcumin is the active agent of the turmeric plant (*Curcuma longa*, turmeric, saffron root, turmeric). Turmeric is a perennial herbaceous plant with yellow flowers and large leaves that belongs to the ginger family and is widely grown in China and India.[11,12] Curcumin has been used as a coloring agent in many foods, cosmetics and drugs, and is known to have antioxidant, anticarcinogenic, antiatherogenic and anti-inflammatory actions in addition to its iron-chelating effects.[11,12] Its anti-inflammatory effects (as an inhibitor of NF-κB) have attracted increasing attention in recent years. Curcumin forms Fe^{+3}-curcumin complexes by binding Fe^{+3}, which increases its known effects. Its chemical binding capacity is greater for Fe^{+3} than Fe^{+2}, depending on the dose and time of application.[10]

The present study investigates the impact of curcumin, alone and in combination with deferoxamine, on cell proliferation and/or implant growth in endometriotic tissues in in vivo experiments, with the aim of establishing the basis for a novel therapeutic strategy for the treatment of endometriosis.

Material and methods

The animals and surgical procedures

After the experimental procedures were approved by the local animal ethics committee, 30 female albino Wistar rats (12 weeks old, weighing 200–250 g) were obtained from the Experimental Animal Center of Trakya University. The initial body weights of the animals were measured. All the animals underwent ovariectomies (to eliminate estrogen from the ovaries) under ketamine- and xylazine-induced anesthesia (respectively: Alfamine, Alfasan International BV, Woerdam, The Netherlands; and Rompun, Bayer AG, Leverkusen, Germany). Next, 21-day release 0.1-mg β-estradiol 17-valerate pellets (E-203, Innovative Research of America, Sarasota, USA) were placed intraperitoneally. All the animals were examined at the same stage of the estrous cycle by waiting 5 days after this procedure. Thereafter, an experimental model of endometriosis was created surgically using the method described by Rezende et al.[4] Briefly, to create the experimental model, an approximately 1-cm-long section of the uterus was removed, primarily from the right horn of the uterus 1 cm above the cervix, and the cylinder-shaped section of the uterus was flattened. The myometrium was split from the endometrium under a microscope, and the dimensions of the 4 endometrial implants thus obtained were measured. Four implants were placed between the peritoneum and muscle. Ten days after this process, 2 test animals were checked, and it was confirmed that the endometriosis model had been created macroscopically and microscopically.

After the establishment of surgically induced endometriosis, the animals were grouped as follows:

Group A was administered injectable water via an intraperitoneal (i.p.) route for 3 days, and then dimethylsulfoxide (DMSO, Merck KGaA, Darmstadt, Germany) via an intragastric (i.g.) route for 20 days, starting on postoperative day 10 of the endometriosis model.

Group B was administered 100 mg/kg curcumin (Sigma-Aldrich Corporation, St. Louis, USA) per day via an i.g. route. The curcumin was dissolved in DMSO and administered for 20 days, starting on postoperative day 10 of the endometriosis model.

Group C was administered 100 mg/kg deferoxamine (Desferal 0.5 g vials, Novartis AG, Istanbul, Turkey) dissolved in injectable water at 6-hour intervals for 3 days via an i.p. route. Then 100 mg/kg curcumin per day was administered by an i.g. route for 20 days, starting on postoperative day 10 of the endometriosis model.

A total of 30 animals (n = 10 each group) were used to develop the endometriosis model. Three animals in group A, 1 animal in group B and 3 animals in group C died at different stages of the experimental protocol. In addition, 2 animals in group B did not display any sign of ectopic endometriosis at the end of the curcumin treatment. These animals were therefore excluded from the study and 7 animals were used from each group.

After administering the agents, the body weights of the animals were measured, biopsy materials were obtained under ketamine/xylazine anesthesia, and the sizes of the endometriotic-like implants were again measured.

Histological and immunohistochemical techniques

All eutopic and ectopic materials were fixed in 10% formaldehyde and embedded in paraffin. The samples were then cut into 5 μm sections and stained with hematoxylin and eosin (H & E) to microscopically confirm whether a model of endometriosis had been created. For immunohistochemical confirmation of the ectopic areas, a monoclonal mouse cytokeratin-7 antibody (5F282:sc-70936, Santa Cruz Biotechnology Inc., Dallas, USA) was used.

For the immunohistochemical procedures, the slides were deparaffinized in toluene and rehydrated in a graded alcohol series. For antigen retrieval, the slides were boiled for 15 min in a 10-millimolar citrate buffer (pH 6). Subsequently, the sections were immersed in 3% hydrogen peroxide for 5 min. To block non-specific staining, the slides were incubated in a humidified chamber with 5% normal horse serum (Vector Laboratories Inc., Burlingame, USA) in Tris-buffered saline (TBS) for 30 min at room temperature. The excess serum was drained and the sections were incubated with a monoclonal mouse PCNA antibody (F-2, sc-25280, 1 : 300 dilution in TBS; Santa Cruz Biotechnology Inc.) and a monoclonal mouse cytokeratin-7 antibody (Santa Cruz Biotechnology Inc.) at room temperature for 1 h. Biotinylated horse anti-mouse antibody (Vector Laboratories Inc.) was added at a 1 : 400 dilution to both primary antibodies for 30 min. The sections were then incubated with a streptavidin-biotin-peroxidase kit (Vector Laboratories Inc.), and the resultant immunoreactivity was visualized using 3,3-diaminobenzidine tetrahydrochloridedihydrate (DAB; Vector Laboratories Inc.). After the sections were slightly counterstained with hematoxylin, they were mounted with coverslips.

The intensity of proliferating cell nuclear antigen (PCNA) immunoreactivity was evaluated in a semi-quantitative manner using the intensity categories and procedure suggested by Seval et al.: "0, no staining; 1+, weak but detectable staining; 2+, moderate or distinct staining; and 3+, intense staining. For each tissue, a histological score (HSCORE) value was derived by calculating the sum of the percentages of cells that stained in each intensity category and then multiplying that value by the weighted intensity of the staining using the formula HSCORE = $\Sigma Pi(i + 1)$, where i represents the intensity scores and Pi is the corresponding percentage of cells".[13] Five randomly selected areas on each slide were evaluated under a light microscope at ×200 magnification at different times by 2 investigators who were blind as to the tissue types and sources, and the percentage of cells at each intensity within these areas was determined. The mean value of the 2 investigators' scores was the basis for the statistical calculations.

Atomicabsorption spectrometry (AAS) technique

Blood taken from the hearts of the animals was kept at –80°C, and the blood iron levels were measured using an atomic absorption spectrophotometer.

Iron (Fe) atomic absorption standard solution (1000 mg/L) was used (Inorganic Ventures, Christiansburg, USA). The Fe concentrations were determined using a Perkin Elmer AAnalyst 800 Atomic Absorption Spectrophotometer (AAS, International Equipment Trading Ltd., Mundelein, USA) with a deuterium lamp and an air/acetylene burner.

The instrumental parameters of the iron element were as follows:

– the hollow cathode lamp was operated at 5 mA for Fe;
– working wavelength: 248.3 nm;
– slit width: 0.2 nm

One milliliter serum samples obtained from the blood samples stored at –80°C were immersed in water at room temperature until they began to appear homogeneous; a wet solubilization method was then applied. The mineral acids and oxidizing acids used in this method are effective substances for solubilizing both organic and inorganic substances. Solubilization occurs because the acids remove the matrix from the samples. For this purpose, 2 mL of an $HClO_4$-HNO_3 acid mixture (1 : 6) was added to the samples, and these samples were incubated in a sealed polyethylene container overnight. This process produced an exact solubilization of the iron content with the oxidant. The solubilized serum samples were filtered into polyethylene bottles using filter paper, and the filtrates were diluted to a volume of 10 mL using distilled water. A blank solution was obtained using the same procedure. The iron in the solubilized serum samples was determined using the atomic absorption spectrophotometer. A standard addition technique was used for the AAS determination due to potential interference present in the tested sample.

Statistical analysis

The statistical analysis was carries out using SPSS 11 software (SPSS Inc., Chicago, USA). The results were expressed as mean ± standard deviation (SD). In all cases, $p < 0.05$ was considered statistically significant. To evaluate statistically significant differences between the 3 groups, the initial and final body weights were assessed using a paired t-test. An ANOVA test was used to compare the endometriotic-like implants sizes, serum iron levels and PCNA immunoreactivity data in each group.

Results

Animal weights, measurement of iron in the blood and endometriotic-like implant size

The body weights of the animals were measured by the same person using the same scale. No significant differences were found between the initial and final weights in any of the 3 groups (Table 1).

The blood iron level measurements were repeated 3 times during the experiment. The blood iron levels in the 3 groups were not significantly different ($p = 0.991$; Table 2).

When the measurements of the endometriotic-like implants at the beginning of the experiment were compared, no significant differences were observed between the 3 groups ($p = 0.762$; Table 1). At the end of the experiment, however, the endometriotic-like implant sizes were

Table 1. Comparison of animal body weights and endometriotic-like implant sizes before and at the end of the experiment

Parameters	Group a (n = 7)	Group b (n = 7)	Group c (n = 7)
Initial body weight (g)	211.85 ± 12.32	214.57 ± 6.67	193.42 ± 15.61
Final body weight (g)	211.28 ± 12.10	219.28 ± 6.1	201.28 ± 23.61
Endometriotic-like implant size before experiment (mm^3)	10.98 ± 2.69	10.56 ± 1.75	11.37 ± 1.23
Endometriotic-like implant size at the end of the experiment (mm^3)	32.89 ± 5.79	23.33 ± 5.41*	23.23 ± 2.95*

* compared with group a, $p < 0.05$. Values are presented as mean ± SD.

Table 2. Blood iron levels and proliferating cell nuclear antigen (PCNA) immunoreactivity results

Parameters	Group a (n = 7)	Group b (n = 7)	Group c (n = 7)
Blood iron (mg/L)	3.23 ± 0.96	3.22 ± 0.48	3.18 ± 0.40
PCNA immunoreactivity in eutopic endometrium	111.43 ± 32.50	100.00 ± 30.35	91.25 ± 28.63
PCNA immunoreactivity in ectopic endometrium	106.83 ± 23.41	69.00 ± 26.32*	67.00 ± 15.25*

* compared with group a, $p < 0.05$. Values are presented as mean ± SD.

significantly reduced in groups b and c – the curcumin and curcumin + deferoxamine groups – as compared with group a (Table 1).

Histopathological evaluation

In all 3 groups, the eutopic endometrium was observed to have a normal histological appearance (Fig. 1a). The ectopic endometrium was characterized by the coexistence of both glands and stromal structures and a remarkable surrounding fibrotic connective tissue. Areas within the ectopic endometrium were found to be particularly well vascularized. Glandular epithelia were observed to have an appearance similar to that of the eutopic epithelial glands (Fig. 1b).

Cytokeratin-7 antibodies in the eutopic and ectopic endometrium exhibited immunoreactivity in the glandular epithelial cells (Figs. 1c, 1d).

Immunohistochemical evaluations

PCNA immunoreactivity was used to assess the proliferation rate of epithelial cells in in the eutopic and ectopic endometria of the control animals, the curcumin-treated

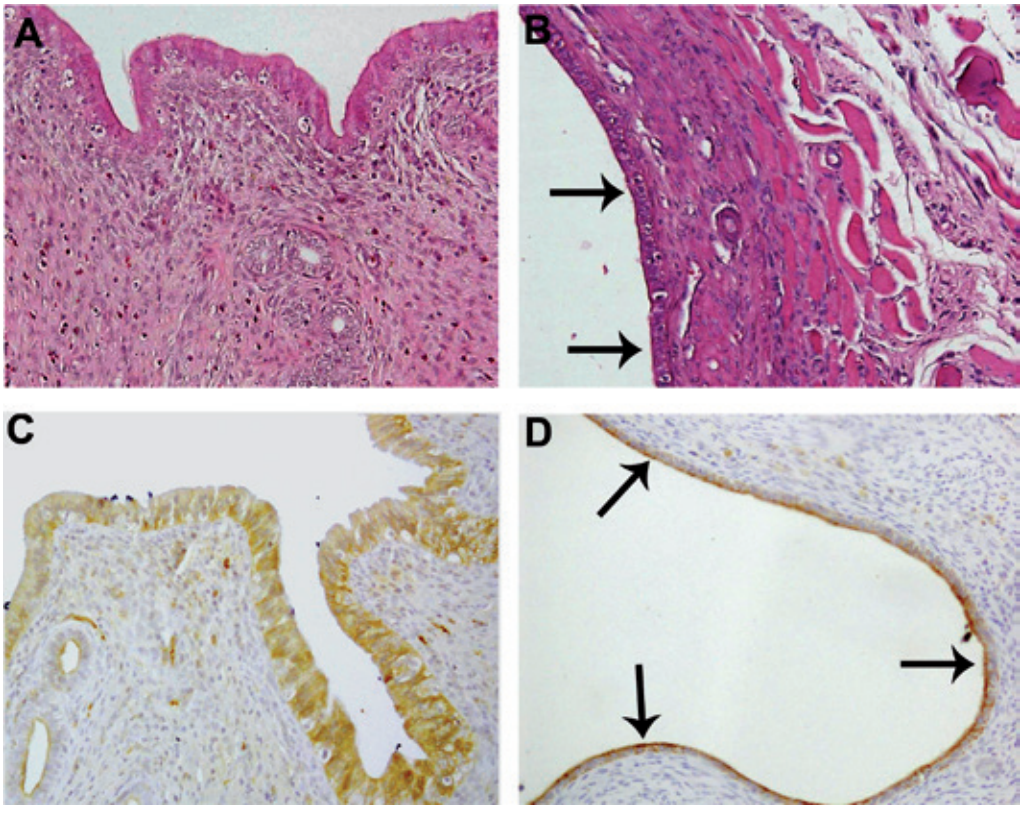

Fig. 1. H & E and cytokeratin-7 evaluation. Eutopic and ectopic (arrows) endometria stained with H & E are shown in a and b, respectively. Cytokeratin-7 immunoreactivity in endometrial glandular cells in eutopic and ectopic (arrows) endometria is shown in c and d, respectively. Magnification x200

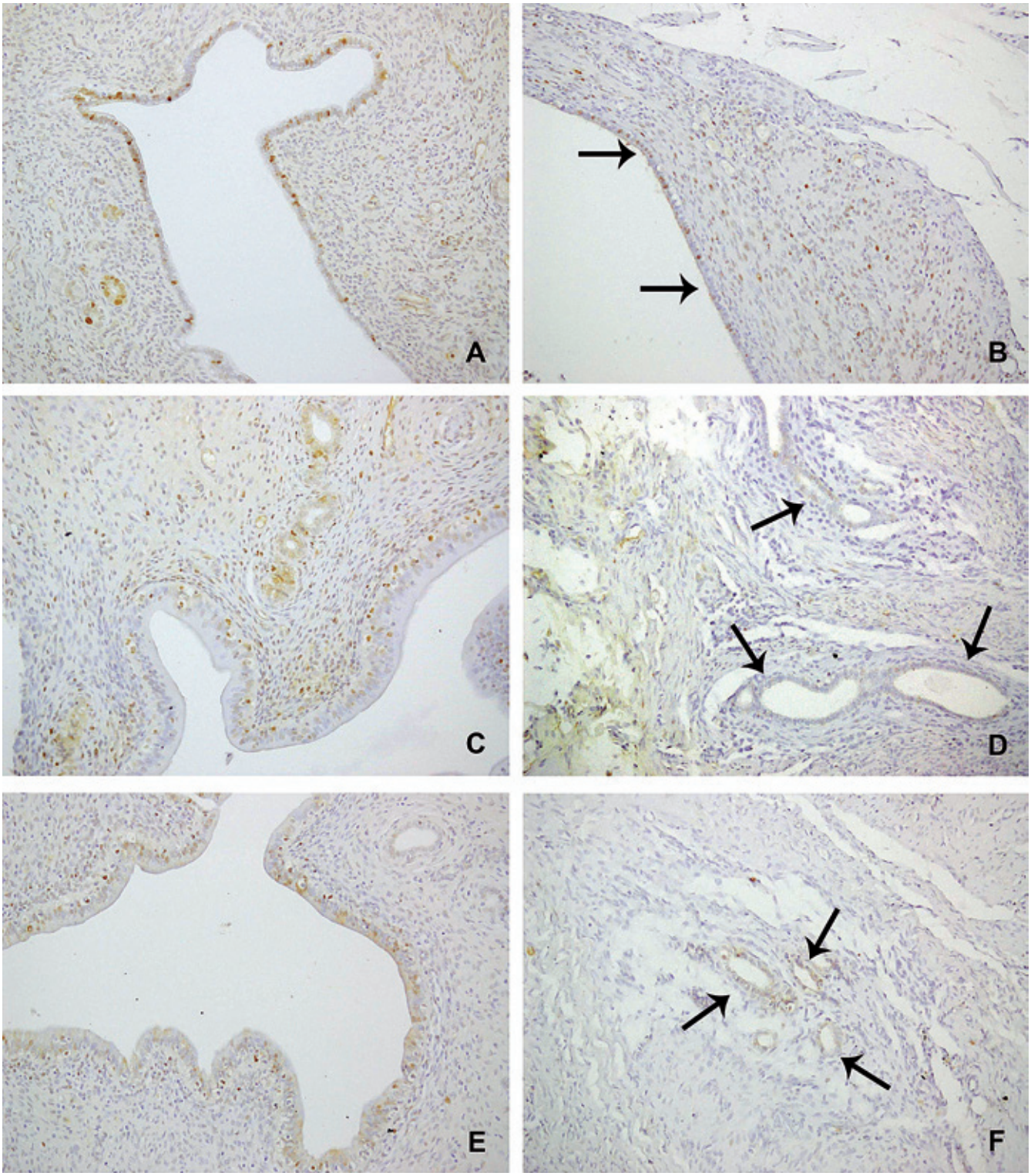

Fig. 2. Proliferating cell nuclear antigen (PCNA) immunoreactivity in eutopic (left column; a, c and e) and ectopic (right column; b, d and f) endometria. No statistically significant differences were found among the groups a, b or c in eutopic endometria based on PCNA immunoreactivity. However, in ectopic endometria (arrows), PCNA immunoreactivity was significantly higher in group a (b) than in group b (d) and c (f). Magnification x200

animals and the curcumin-with-deferoxamine-treated animals. The values are shown in Table 2.

When the PCNA immunoreactivities in the eutopic endometria of groups a, b and c were compared, no significant differences were found among the groups ($p = 0.454$; Figs. 2a, 2c, 2e and Table 2).

In the ectopic endometrium, PCNA immunoreactivity was significantly lower in groups b and c compared with group a ($p = 0.044$ and $p = 0.033$, respectively; Figs. 2b, 2d and 2f; Table 2). A comparison between the ectopic endometria in groups b and c revealed no significant differences ($p = 1.00$; Figs. 2d–f).

Discussion

Endometriosis is an increasingly important disease that affects women of reproductive age and can cause infertility. Interest in this disease continues to grow among investigators, as a cure has yet to be found.[14,15]

In the present study, no differences were observed in the 3 groups' pre- and post-experimental body weights (Table 1), which coincides with other researchers' findings.[16] These results suggest that neither curcumin alone nor curcumin in combination with deferoxamine significantly affects metabolic activity in rats.

Iron levels, which are known to be increased in endometriosis, are considered a possible marker for endometriosis or experimental endometriosis.[5–7,17,18] The present study measured blood iron levels to determine whether they would change with curcumin alone or in combination with deferoxamine in eutopic and/or ectopic endometrial tissues. This study also investigated whether cell proliferation and the sizes of the endometrial implants or the development of experimental endometriosis would be affected in the groups that received curcumin alone or curcumin + deferoxamine.

In the present study, atomic absorption spectrophotometry showed that the blood iron levels were not significantly different between the 2 treatment groups. These results suggest, at least in part, that neither curcumin nor its combination with deferoxamine affect serum iron levels in a rat endometriosis model. However, Langendonckt et al. suggested that serum iron concentrations were higher than in peritoneal fluid using a colorimetric method in women with endometriosis.[18]

In a study by Zhang et al., a similar endometriosis model was created by inserting large endometrial implants. Subsequently, 3 different doses of curcumin (50, 100 and 150 mg/kg) were administered to the animals. Those authors reported that the endometriotic-like implant sizes were significantly decreased in each group.[16] The results of the group administered 100 mg/kg of curcumin were the same as those observed in the curcumin group in the present study. Similarly, Hendarto et al. emphasized that curcumin had reduced the size of endometriotic-like implants in experimental endometriosis in mice.[19] In all 3 studies, including the current findings, curcumin's reduction of the size of endometriotic-like implants is very promising, and warrants further studies aiming toward the development of a novel therapeutic strategy for treatment of endometriosis.

Weiser et al. suggested that curcumin decreases cell proliferation, suppresses the secretion of vascular endothelial growth factor (VEGF) and causes apoptosis in eutopic endometrial cells in women with and without endometriosis.[20] Recent studies have revealed that curcumin nearly reversed the activity of matrix metalloproteinases (MMP)-2, MMP-3 and MMP-9, and that increasing MMP-2, MMP-3 and MMP-9 levels in patients with endometriosis exacerbated the disease.[21–23] In addition, curcumin, which is also an inhibitor of NF-κB can block all the potential effects of NF-κB by activating various pathways and increasing the secretion of different proteins in endometriosis.[24–27]

Defrère et al. found that the proliferative activity of epithelial cells in the ectopic endometrium decreased when deferoxamine was applied to nude mice with experimental endometriosis.[6] They concluded that an iron overload does not affect the occurrence of new lesions. Moreover, it may cause endometriosis to spread by increasing epithelial cell proliferation in these lesions.[6] According to this view, which is parallel to the present authors' ideas, iron-chelating treatments may be useful for endometriosis, reducing the proliferation of lesions by preventing iron loading in the pelvic cavity.[17] Although the present authors did not observe further improvement in experimental endometriosis using the curcumin + deferoxamine combination as compared with curcumin alone, future studies examining increases in the dose and timing of this combination may reveal possible additive effects to a significant level when compared with curcumin alone.

Conclusions

In conclusion, to the best of the authors' knowledge, this is the first experimental endometriosis study demonstrating the combined effect of curcumin and deferoxamine. The findings suggest that the combined use of different iron-chelating agents may lead to a reduction in endometrial implant sizes and cell proliferation when used to treat endometriosis. However, further studies from many different perspectives are required.

References

1. Kizilay G, Cakmak H, Arici A. Endometriosis and implantation. *Balkan Med J*. 2007;24:90–97.
2. Ihara T, Uchiide I, Sugamata M. Light and electron microscopic evaluation of antileukotriene therapy for experimental rat endometriosis. *Fertil Steril*. 2004;81:819–823.
3. Sharpe-Timms KL. Using rats as a research model for the study of endometriosis. *Ann N Y Acad Sci*. 2002;955:318–327.
4. Rezende CAL, Silva AL, Junior JLS, Gobbi H, Martins MMF. Experimental model for endometriosis-comparative histological study between the ectopic and eutopic endometrium. *Acta Cír Bras*. 1997;12:226–230.
5. Lousse JC, Defrere S, Van Langendonckt A, et al. Iron storage is significantly ıncreased in peritoneal macrophages of endometriosis patients and correlates with iron overload in peritoneal fluid. *Fertil Steril*. 2009;9:1668–1675.
6. Defrere S, Van Langendonckt A, Vaesen S, et al. Iron overload enhances epithelial cell proliferation in endometriotic lesions induced in a murine model. *Hum Reprod*. 2006;21:2810–2816.
7. Van Langendonckt A, Casanas-Roux F, Eggermont J, Donnez J. Characterization of iron deposition in endometriotic lesions induced in the nude mouse model. *Hum Reprod*. 2004;19:1265–1271.
8. Langlois A, Bietiger W, Mandes K, et al. Overexpression of vascular endothelial growth factor in vitro using deferoxamine: A new drug to increase islet vascularization during transplantation. *Transplant Proc*. 2008;40:473–476.

9. Wu MH, Chen KF, Lin SC, Lgu CW, Tsai SJ. Aberrant expression of leptin in human endometriotic stromal cells is induced by elevated levels of hypoxia inducible factor-1α. *Am J Pathol*. 2007;70:590–598.
10. Srichairatanakool S, Thephinlap C, Phisalaphong C, Porter JB, Fucharoen S. Curcumin contributes to in vitro removal of non-transferrin bound iron by deferiprone and desferrioxamine in thalassemic plasma. *Med Chem*. 2007;3:469–474.
11. Hatcher H, Planalp R, Cho J, Torti FM, Torti SV. Curcumin: From ancient medicine to current clinical trials. *Cell Mol Life Sci*. 2008;65: 1631–1635.
12. Kim KH, Lee EN, Park JK, et al. Curcumin attenuates TNF-α-induced expression of intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and proinflammatory cytokines in human endometriotic stromal cells. *Phytother Res*. 2012;26:1037–1047.
13. Seval Y, Cakmak H, Kayisli UA, Arici A. Estrogen-mediated regulation of p38 mitogen-activated protein kinase (MAPK) in human endometrium. *J Clin Endocrinol Metab*. 2006;91:2349–2357.
14. Ricci AG, Olivares CN, Bilotas MA, et al. Natural therapies assessment for the treatment of endometriosis. *Hum Reprod*. 2013;28:178–188.
15. Nenicu A, Körbel C, Gu Y, Menger MD, Laschke MW. Combined blockade of angiotensin II type 1 receptor and activation of peroxisome proliferator-activated receptor-γ by telmisartan effectively inhibits vascularization and growth of murine endometriosis-like lesions. *Hum Reprod*. 2014;29:1011–1024.
16. Zhang Y, Cao H, Hu YY, Wang H, Zhang CJ. Inhibitory effect of curcumin on angiogenesis in ectopic endometrium of rats with experimental endometriosis. *Int Jour of Mol Med*. 2011;27:87–94.
17. Defrère S, Lousse JC, González-Ramos R, Colette S, Donnez J, Van Langendonckt A. Potential involvement of iron in the pathogenesis of peritoneal endometriosis. *Mol Hum Reprod*. 2008;14:377–85.
18. Van Langendonckt A, Casanas-Roux F, Donnez J. Iron overload in the peritoneal cavity of women with pelvic endometriosis. *Fertil Steril*. 2002;78:712–718.
19. Hendarto HHH, Kuswojo HKK, Sa'adi AAS, Ramelan WWR, Sudiana IKIKS. The role of curcumin supplementation on implant growth and fertilization result of experimental endometriosis in mice. *Fertil Steril*. 2010;94:205.
20. Wieser F, Yu J, Park J, Sidell N, Taylor RN. Curcumin suppresses angiogenesis, cell proliferation and induces apoptosis in an in vitro model of endometriosis. *Fertil Steril*. 2007;88:204–205.
21. Jana S, Rudra DS, Paul S, Snehasikta S. Curcumin delays endometriosis development by inhibiting MMP-2 activity. *Indian J Biochem Biophys*. 2012;49:342–348.
22. Jana S, Paul S, Swarnakar S. Curcumin as anti-endometriotic agent: Implication of MMP-3 and intrinsic apoptotic pathway. *Biochem Pharmacol*. 2012;83:797–804.
23. Swarnakar S, Paul S. Curcumin arrests endometriosis by downregulation of matrix metalloproteinase-9 activity. *Indian J Biochem Biophys*. 2009;46:59–65.
24. Poylin V, Fareed MU, O'Neal P, et al. The NF-Kappaβ inhibitor curcumin blocks sepsis-induced muscle proteolysis. *Mediators Inflamm*. 2008;2008:ID:317851.
25. Kim YS, Ahn Y, Hong MH, et al. Curcumin attenuates inflammatory responses of TNFα-stimulated human endothelial cells. *J Cardiovasc Pharmacol*. 2007;50:41–49.
26. Veillat V, Lavoie CH, Metz CN, Roger T, Labelle Y, Akoum A. Involvement of nuclear factor-κB in macrophage migration inhibitory factor gene transcription up-regulation induced by interleukin-1β in ectopic endometrial cells. *Fertil Steril*. 2009;91:2148–2156.
27. Reinaldo GR, Langendonckt AV, Defrere S, et al. Involvement of the nuclear factor-κB pathway in the pathogenesis of endometriosis. *Fertil Steril*. 2010;94:1985–1994.

The role of 17β-estradiol metabolites in chromium-induced oxidative stress

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Abstract

Background. The increasing incidence of estrogen-dependent breast cancer and the presence in the environment of a large number of factors that interact with estrogen receptors have sparked interest in chemical influences on estrogen-dependent processes. In a previous work, the authors examined the interaction of estradiol with chromium. In the present article the importance of estradiol biotransformation in these interactions is investigated. There is no information in the available literature about the role of metabolites in exposure to chromium. It seems important because estradiol metabolites have various carcinogenic abilities and their formation during biotransformation could be increased or decreased by environmental enzyme inducers or inhibitors. The metabolites could play a detoxifying role or create a toxic synergism in free radical processes induced by chromium VI (CrVI).

Objectives. The aim of this study was to evaluate the influence of 2 17β-estradiol metabolites – 4-hydroxyestradiol (4-OHE2) and 16α-hydroxyestrone (16α-OHE1) – in conditions of oxidative stress caused by CrVI.

Material and methods. Human blood, erythrocytes or mitochondria isolated from human placentas after natural deliveries were used in the experiments. The influence of CrVI, 4-OHE2 and 16-OHE1 on thiobarbituric acid reactive substances (TBARS), the hydroxyl radical (•OH), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione-S-transferase (GST), and the interactions of the metabolites exposed to chromium expressed by these factors were examined.

Results. 4-OHE2 reduced the level of TBARS induced by CrVI in mitochondria ($p < 0.05$) and in erythrocytes ($p < 0.05$), and increased SOD activity ($p < 0.05$). 16α-OHE1 increased the activity of GST in erythrocytes exposed to CrVI ($p < 0.05$).

Conclusions. The metabolites do not have toxic interactions with CrVI. On the contrary, they exhibited a protective effect. The mechanism of protection varied: 4-OHE2 decreased TBARS and increased SOD activity, while 16α-OHE1 increased GST activity.

Key words: oxidative stress, interactions, chromium VI, 4-hydroxyestradiol, 16α-hydroxyestrone

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The increasing incidence of estrogen-dependent breast cancer has sparked researchers' interest in the influence of environmental factors on estrogen-dependent processes. The interaction of xenobiotics with estrogens has been widely studied in case of environmental toxins that interact with estrogen receptors (xenoestrogens). However, chromium is recognized as a metalloestrogen, and there are only a few reports in the available literature on chromium/estrogen interaction.[1–3] There is no information about the role of estradiol metabolites in exposure to chromium. The problem seems important because the metabolites have various carcinogenic abilities, and their formation during estradiol biotransformation could be increased or decreased by environmental enzyme inducers or inhibitors. The main biotransformation pathway of estradiol is hydroxylation. The final products of C-4, C-16 or C-2 hydroxylation are 4-hydroxyestradiol (4-OHE_2), 16-α-hydroxyestrone (16α-OHE_1) or 2-metoxyestradiol (2-$MeOE_2$) respectively. The role of these metabolites in carcinogenesis is unknown; however, there are some reports on the presence of 4-OHE_2-adducts in breast cancer tissue and on the anticancer activity of 2-$MeOE_2$.[4]

Chromium is widely present in the environment. Its toxicity depends on the valence. The International Agency for Research on Cancer (IARC) classifies chromium (VI) as a proven human carcinogen.Interactions between estrogens and carcinogenic factors need special attention because estrogen itself possesses carcinogenic activity, so synergistic effects with carcinogenic chromium could be very dangerous. Because exposure to metals is widespread, an elucidation of their roles in the etiology and development of hormone-related diseases such as breast cancer may have significant implications in risk reduction and disease prevention. Chromium compounds are able to generate free radicals, which have an indisputable role in carcinogenesis.[5] Estrogens in general act as free-radical scavengers, but also demonstrate prooxidative activity. Not only chromium but also estrogens are present in environment. The metabolites of estrogen used in contraception or hormonal replacement therapy are found in ground water and wastewater. They also circulate in the environment via communal waste, increasing the risk of hormonally active compounds interacting with xenobiotics. A number of publications describe the presence of active hormonal compounds originating from waste or breeding farms and their influence on the environment.[6,7]

The authors' previous work demonstrated the protective role of 17β-estradiol (E_2) in chromium-induced lipid peroxidation in human erythrocytes, but in the mitochondrial membranes of human placenta, the opposite phenomenon was observed: 17β-estradiol increased CrVI-induced lipid peroxidation in mitochondria, so a toxic interaction was noted.[3] The mechanism of this interaction partially correlated with hydroxyl radical (•OH) formation, since E_2 increased •OH generation in mitochondria exposed to low (0.05 and 0.5 μg/mL) CrVI concentrations. These interactions were not observed for superoxide dismutase (SOD) activity.

It seems valid to assess the role of estradiol metabolites in the processes outlined above. Environmental toxins often change the biotransformation pathways, acting as inductors or inhibitors of particular enzymes. The aim of the present study was to evaluate the influence of estradiol metabolites 4-OHE_2 and 16α-OHE_1 on mitochondria and erythrocytes when exposed to CrVI. There are no data on that topic at present. It is known that the biotransformation of estradiol plays important role in carcinogenesis, and the level of some metabolites, e.g. 16α-OHE_1,is increased in women with breast cancer.[8] In this work the influence of 16α-OHE_1 and 4-OHE_2 on the lipid peroxidation level (TBARS) and hydroxyl radical generation (•OH level), elements of the enzymatic and non-enzymatic antioxidant barrier such as superoxide dismutase (SOD), and detoxifying capacity measured by GST activity was tested. All the experiments were conducted on human cells in vitro: erythrocytes of blood or mitochondria obtained from placenta.

Material and methods

The study was conducted on an in vitro model, using human cell erythrocytes and mitochondria. The study was approved by the Wroclaw Medical University Ethics Committee (permit KB-292/2013). Mitochondria were isolated from human placenta, obtained after natural deliveries from the Clinic of Reproduction and Obstetrics, Wroclaw Medical University (Poland), by a previously described method.[9] The isolated mitochondrial pellet was suspended in 50 mMTris-HCl buffer (pH 7.4), and the level of protein was measured usingthe Lowry method.[10] The suspension was portioned into Eppendorf tubes (1 mL) and stored at –80°C, no longer than 3 months before use. The mitochondrial suspension was used for the evaluation of lipid peroxidation (TBARS), hydroxyl radical generation (•OH), superoxide dismutase (SOD) and glutathione-S-transferase (GST) activities.

Fresh human blood taken on sodium citrate for SOD, GST and TBARS determination was obtained from healthy donors at Wroclaw University Hospital. The erythrocytes were isolated by whole blood centrifugation for 10 min at room temperature (3000 rpm). The plasma and white blood cells were then removed and the resulting pellet of erythrocytes was washed 3 times in phosphate buffered saline (PBS). The erythrocytes were suspended in PBS at a 10% concentration for the SOD, GST and TBARS measurements.

Potassium dichromate ($K_2Cr_2O_7$) (POCH S.A., Gliwice, Poland) was used as the CrVI source. The CrVI concentrations used in the experiments were 0.05 μg/mL, 0.5 μg/mL and 1.0 μg/mL.

Estradiol metabolites 16α-hydroxyestrone (3,16α-Dihydroxy-1,3,5(10)-estratrien-17-one) (Steraloids Inc., New-

port, USA) and 4-hydroxyestradiol (Estra-1,3,5(10)-triene-3,4,17ß-triol) (Sigma-Aldrich Corp., St. Louis, USA) were dissolved in 96% ethanol (P.P.H. POCh S.A.) to a concentration of 10 mM stock solution and used diluted in PBS for every experiment at concentrations of 1.0 μM, 5.0 μM and 10.0 μM.

Appropriate controls were assayed in parallel to the samples. All the experiments were performed gradually, using different sources of red blood cells and mitochondria in each exposure scenario.

Determination of TBARS concentration

In mitochondria

The mitochondrial suspension (1 mL) was incubated in a water bath with a shaker at 37°C for 30 min with 30 μL of CrVI, 16α-OHE_1, 4-OHE_2 or a mixture (to assess interaction). The incubation was stopped by adding 0.5 mL of 20% trichloroacetic acid (TCA). Then 1.5 mL of 0.67% thiobarbituric acid (TBA) and 30 μL of 1% buthylhydroxytoluene (BHT) were added and the samples were heated at 95°C for 15 min. The cooled mixture was centrifuged to pellet the protein. The TBARS concentration was measured spectrophotometrically at 535 nm and expressed as nM per mg of mitochondrial protein, using the molar absorption coefficient $1.56 \times 105\ M^{-1}\ cm^{-1}$.[11,12]

The effects of metabolite interactions with chromium VI on TBARS concentration were examined in every concentration: 4-OHE_2 and 16α-OHE_1 at 1.0 μM, 5.0 μM and 10.0 μM; CrVI at 0.05 μg/mL, 0.5 μg/mL and 1.0 μg/mL. First the mitochondria were incubated at 37°C for 30 min with 16α-OH or 4-OHE_2 at the appropriate concentration, then chromium VI was added to the sample and the incubation was continued at 37°C for 30 min. Then the TBARS concentration was measured by the method described above.

In erythrocytes

The compounds being studied were added in 20 μL quantities to 2 mL of erythrocyte suspension prepared as described above, and the samples were incubated at 37°C for 0.5 h in a water bath with shaker. The TBARS concentration was then measured as described by Stocks et al.[13] The concentration of lipid peroxidation products was read from a standard curve, prepared with tetra ethoxypropane and expressed in nM/g Hb. Hemoglobin in the blood was determined using Drabkin's reagent.

Determination of hydroxyl radical generation

The mitochondrial suspension (0.5 mL) was incubated at 37°C (15 min) with 15 μL of 1% t-BOOH, 0.5 mL of 20 mM deoxyribose and either 4-OHE_2, 16α-OHE_1 or CrVI; and to assess interactions, 4-OHE_2 combined with CrVI, or 16α-OHE_1 combined with CrVI. After incubation, the samples were centrifuged and 0.8 mL of the supernatant was collected; then 0.5 mL of 2.8% TCA and 0.5 mL of 1% TBA in 0.1 M NaOH were added to the supernatant. The samples were incubated at 100°C for 20 min, then cooled and centrifuged. The level of •OH generation was measured spectrophotometrically at 532 nm and calculated using the molar coefficient of absorption $1.56 \times 105\ M^{-1}cm^{-1}$.[12,14]

Determination of superoxide dismutase activity

Superoxide dismutase activity was measured with a RANSOD kit (Randox Laboratories Ltd., Crumlin, UK) according to the manufacturer's instructions.[15,16] The assay is based on the xanthine oxidase reaction, where superoxide anion product reacts with 2–(4–iodophenyl)–3–(4–nitro–phenol)–5–phenyltetrazolium chloride (INT) to form a red formazan dye measured at a wavelength of 492 nm. The strength of inhibition of formazan dye formation reflects the activity of SOD in the tested sample. The assay mixture, with a final volume of 0.2 mL, included xanthine oxidase, xanthine, INT and the sample. Absorbance was measured at 492 nm after the addition of xanthine oxidase. On the basis of the absorbance increase after 1 min and comparison with the calibration curve, the percentage of reaction inhibition and hence of SOD activity was calculated according to the kit manufacturer's instructions. The results were compared with a sample containing CrVI, prepared in the same way, but without estrogens.

Determination of glutathione-S-transferase activity

Glutathione S-transferase activity was assayed using 1-Chloro-2, 4-dinitrobenzene (CDNB), which is suitable for the broadest range of GST isoenzymes.[17] Upon conjugation of the thiol group of glutathione to the CDNB substrate, there is an increase in absorbance at 340 nm. Solutions of samples containing CrVI, 16α-OHE_1, 4-OHE_2 or mixtures of them were mixed with the substrate on a shaker, and the absorbance was immediately measured at a wavelength of λ = 340 nm on a plate reader (StatFax 2100 Awareness Technology, Block Scientific Inc., Bellport, USA). The increase in absorbance in the tested sample was directly proportional to the GST activity.

In order to evaluate the effect of estradiol metabolites on human placental mitochondria or erythrocytes stimulated with CrVI, the mitochondrial or erythrocyte suspension was preincubated at 37°C for 30 min with appropriate concentrations of the estradiol metabolites (16α-OHE_1 or 4-OHE_2). Then a solution of CrVI was added to the sample and incubation was continued at 37°C for 30 min. Finally,

the level of the factor being tested was measured and compared with controls containing chromium in the appropriate concentration but without estradiol metabolites.

Statistical analyses

All statistical analyses were performed using Student's *t*-test or one-way ANOVA for repeated measurements. Differences between the groups were considered significant at $p < 0.05$. The variability of the distribution was checked with the Lilliefors test. Pearson's correlation coefficient was used to describe correlations. The statistical evaluation of the results was carried out using STATISTICA 10 software (StatSoft Inc., Tulsa, USA).

Results

The effect of CrVI compounds, 4-OHE_2 and 16-αOHE_1 on TBARS and •OH concentration, and on the activities of SOD and GST, both in mitochondria from human placenta and in erythrocytes from blood is presented. In order to better interpret the combined action of CrVI and the metabolites, the effect of the individual compounds (CrVI, 4-OHE_2 or 16-αOHE_1) on selected parameters in the mitochondria and erythrocytes was also presented (Table 1).

It was found that all the concentrations of chromium VI used in the tests (0.05, 0.5 and 1.0 μg/mL) increased the lipid peroxidation measured by TBARS concentration in the mitochondrial and erythrocyte suspension in comparison with equivalent controls (Table 1). The combined action of estrogens and CrVI showed that 4-OHE_2 at a concentration of 1.00 μM significantly ($p < 0.05$) reduced the level of chromium-induced (0.05 and 0.5 μg/mL of CrVI) lipid peroxidation in erythrocytes. Decreases in TBARS concentrations were not observed for the highest chromium concentrations (Table 2).

Unexpectedly, in mitochondria treated with CrVI, the metabolite 4-OHE_2 also reduced the TBARS level ($p < 0.05$) with a clear negative statistically significant dose/response dependency (Pearson correlation coefficient $r = -0.972$ for 0.05 μg/mL CrVI; $r = -0.9494$ for 0.5 μg/mL CrVI; $r = -0.9856$ for 1.0 μg/mL CrVI; $p < 0.001$) (Fig. 1). The statistically significant effect of 4-OHE_2 was observed on lipid peroxidation caused by CrVI at all 3 doses: 0.05 μg/mL, 0.5 μg/mL and 1.0 μg/mL. The largest decrease in TBARS levels was at the highest concentration of 4-OHE_2 (10.0 μM). This is interesting, because the authors' previous report described17-β-estradiol (E_2) having the opposite effect on erythrocytes than on mitochondria exposed to chromium.[3]

The other estradiol metabolite, 16α-OHE_1, in contrast to 4-OHE_2, did not decrease chromium-induced lipid

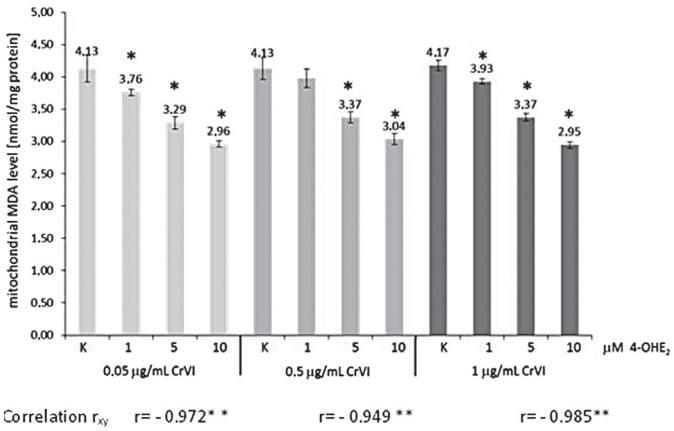

Fig. 1. Effect of 4-OHE2 on mitochondrial TBARS level upon Cr(VI) exposure *Difference significant vs control (K) without 4-OHE2 (containing CrVI in appropriate concentration) ($p < 0.05$) ** statistically significant correlation ($p < 0.05$)

Table 1. The mean levels of the examined parameters in mitochondria and erythrocytes after exposure to CrVI, 4-OHE2 or 16-alfa-OHE1

Compound – concentration	TBARS in mitochondria [nM/mg protein]	TBARS in erythrocytes [nM/g Hb]	•OH in mitochondria [nM/mg protein]	SOD in mitochondria [U/mg protein]	SOD in erythrocytes [U/g Hb]	GST in mitochondrial [U/mg protein]	GST in erythrocytes [U/g Hb]
CrVI [μg/mL]							
Control (0)	2.69 ± 0.22	1.24 ± 0.11	5.52 ± 0.4	84. 5± 8.5	79.5 ± 12.5	0.12 ± 0.02	0.095 ± 0.01
0.05	4.13 ± 0.19*	1.86 ± 0.13*	6.16 ± 0.16*	61.13 ± 11.2*	51.1 ± 6.2*	0.10 ± 0.03	0.080 ± 0.004*
0.5	4.13 ± 0.21*	1.9 2± 0.13*	5.85 ± 0.27	74.98 ± 15.2*	69.16 ± 9.8*	0.096 ± 0.02*	0.080 ± 0.003*
1.0	4.17 ± 0.11*	1.66 ± 0.09	5.59 ± 0.26	81.22 ± 20.1	76.43 ± 11.5	0.090 ± 0.004*	0.081 ± 0.002*
4-OHE_2 [μM]							
Control (0)	2.58 ± 0.12	1.08 ± 0.16	4.82 ± 1.20	125.2 ± 20.3	106.9 ± 15.2	2.29 ± 0.05	0.85 ± 0.22
1.0	3.08 ± 0.40	1.02 ± 0.19	5.23 ± 1.55	160.0 ± 18.3	259.0 ± 17.3*	2.18 ± 0.11	0.47 ± 0.2
5.0	2.89 ± 0.35	1.15 ± 0.22	6.41 ± 1.28	285.0 ± 35.2*	212.2 ± 32.3*	1.88 ± 0.19*	0 .49 ± 0.28
10.0	2.41 ± 0.39	1.48 ± 0.36	6.60 ± 2.15	350.0 ± 46.1*	317.6 ± 47.2*	1.50 ± 0.126*	0.56 ± 0.17
16-α-OHE_1 [μM]							
Control (0)	3.32 ± 0.31	3.4 ± 0.23	8.80 ± 2.4	138.2 ± 18.0	124.0 ± 19.2	1.43 ± 0.15	1.38 ± 0.1
1.0	3.01 ± 0.29	2.8 ± 0.9	8.05 ± 1.9	168.0 ± 23.5	166.4 ± 22.3*	1.37 ± 0.2	1.73 ± 0.04*
5.0	3.72 ± 0.40	2.9 ± 0.8	8.95 ± 0.79	185.0 ± 42.8	125.0 ± 17.2	1.28 ± 0.11	1.71 ± 0.35*
10.0	3.94 ± 1.20	3.25 ± 0.9	8.5 ± 1.25	172.0 ± 79.2	130.0 ± 21.2	1.15 ± 0.12	1.81 ± 0.12*

*$p < 0.05$ as compared with control (0) [CrVI = 0 μg/mL; 4-OHE_2 = 0 μM; 16α-OHE_1 = 0 μM].

Table 2. Effects of 4-OHE2 (1.0; 5.0 and 10 μM) on TBARS levels in erythrocytes exposed to CrVI (0.05, 0.5 and 1.0 μg/mL)

Cr (VI) concentration [μg/mL]	TBARS concentration [nM/gHb]±SD			
	control	4-OHE_2 concentration [μM]		
		1.0	5.0	10.0
0.05	1.86 ± 0.13	1.42* ± 0.12	1.74 ± 0.08	1.99 ± 0.11
0.5	1.92 ± 0.12	1.66* ± 0.11	1.12* ± 0.12	1.76 ± 0.21
1.0	1.66 ± 0.09	1.60 ± 0.23	1.61 ± 0.19	1.62 ± 0.10

* difference significant as compared with the control without 4-OHE_2 (containing only CrVI) ($p < 0.05$).

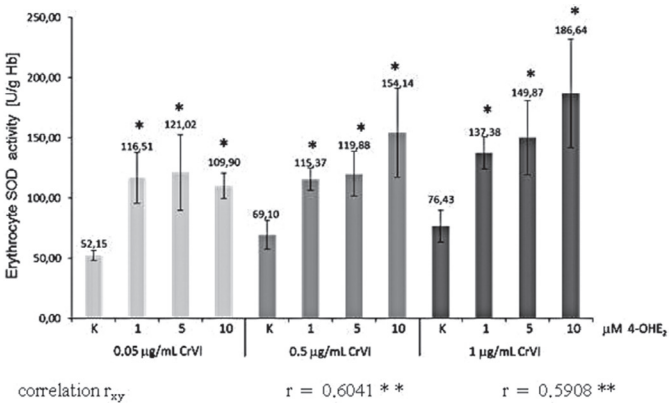

Fig. 2. Effect of 4-OHE2 on erythrocyte SOD activity upon Cr(VI) exposure *difference significant vs control (K) without 4-OHE2 (containing CrVI in appropriate concentration) ($p < 0.05$) ** statistically significant correlation ($p < 0.05$)

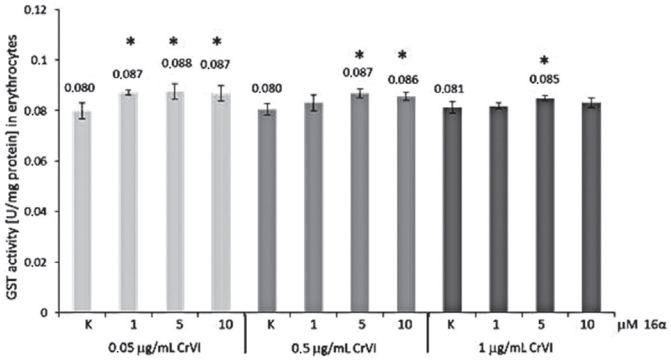

Fig. 3. Influence of 16α-OHE1 on erythrocyte GST activity upon Cr(VI) exposure *difference significant vs control (K) without 16α-OHE1 (containing CrVI in appropriate concentration) ($p < 0.05$)

peroxidation in erythrocytes and mitochondria (data not shown). It could therefore be concluded that 16α-OHE_1 did not participate in the decrease in lipid peroxidation shown by E_2 exposed to CrVI in the authors' previous study.[3] It appears that the positive effect of E_2 could be increased by its biotransformation to 4-OHE_2, not to 16α-OHE_1.

The studies of the joint effects of 4-OHE_2 or 16-αOHE_1 and CrVI in mitochondria revealed that neither of the metabolites in combination with CrVI influenced •OH generation (data not shown). These results suggest that the effect of the examined compounds on free radical processes engages a mechanism that is not dependent on the •OH level.

As to the enzyme activity studies, CrVI in doses of 0.05 μg/mL and 0.5 μg/mL decreases SOD in a statistically significant way in comparison to equivalent controls, in both erythrocytes and in mitochondria ($p < 0.05$) (Table 1). The joint influence of metabolites and CrVI on SOD revealed the clear stimulative effects of all concentrations of 4-OHE_2 on SOD activity in erythrocytes exposed to CrVI; dose/response dependency was observed for CrVI in concentrations of 1.0 μg/mL and 0.5 μg/mL ($r = 0.5908$ and $r = 0.6041$ respectively; $p < 0.05$).

Most of the data showed a statistically significant increase in SOD activity treated with 4-OHE_2 and CrVI in comparison to controls where erythrocytes were exposed to chromium alone (Fig. 2). As in erythrocytes, 4-OHE_2, significantly increased SOD activity in mitochondria exposed to CrVI (Table 3), but without clear dose/response dependency. No effects of 16α-OHE_1 on SOD activity in erythrocytes or mitochondria were observed (data not shown).

In all the doses used, chromium VI decreased GST activity in erythrocytes and in mitochondria ($p < 0.05$) in comparison to equivalent controls (0) (Table 1). In erythrocytes exposed to 0.05 μg/mL, 0.5 μg/mL and 1.0 μg/mL of CrVI, the metabolite 16α-OHE_1 caused a clear increase in GST activity (Fig. 3). The most significant effect was observed when erythrocytes were exposed to the lowest dose of CrVI (0.05 μg/mL). The effect on mitochondria

Table 3. Effects of 4-OHE2 (1.0, 5.0 and 10 μM) on mitochondrial SOD activity in exposure to CrVI (0.05, 0.5 and 1.0 μg/mL)

CrVI concentration [μg/mL]	SOD activity [U/mg protein] ± SD			
	control	4-OHE_2 concentration [μM]		
		1.0	5.0	10.0
0.05	61.13 ± 11.2	126.37* ± 24.96	93.07 ± 13.02	99.05* ± 558
0.5	74.98 ± 15.2	218.87 ± 102.19	221.96* ± 76.32	138.42* ± 19.37
1.0	81.22 ± 20.1	129.48* ± 14.32	152.62* ± 15.13	150.19* ± 36.32

* difference significant as compared with the control without 4-OHE_2 (containing only CrVI) ($p < 0.05$).

was not significant (data not shown). No influence of 4-OHE_2 on GST activity was observed in erythrocytes or mitochondria exposed to CrVI.

To summarize, it was observed that neither 4-OHE_2 and 16α-OHE has toxic reactions to CrVI; on the contrary, they even show positive, protective effects in mitochondria and in erythrocytes exposed to CrVI: 4-OHE_2 decreases lipid peroxidation in mitochondria and erythrocytes, increasing SOD activity. The positive role of

16α-OHE_1 involves a different mechanism, influencing phase-II detoxification by stimulating glutathione transferase activity.

Discussion

The role of estradiol in oxidative stress is prominent because it demonstrates free radical scavenging activities. This activity is enabled by the presence of a hydroxyl phenolic group that acts as a proton donor in chemical reactions.[18] The estrogenic radical formed in this deprotonation is stabilized by electron delocalization inside the aromatic ring. On the basis of this mechanism, it can be concluded that the estradiol metabolites 4-OHE_2 and 16α-OHE_1 should demonstrate similar properties, since both these compounds contain an aromatic ring substituted with a hydroxyl group. It follows that 4-OHE_2 should be an even stronger free-radical scavenger in cells than in the substrate, since it contains a second additional phenolic group typical of catecholestrogens. The catecholic structure favors transformation to o-quinone and semiquinone radicals, which are less toxic than the hydroxyl radical. The catecholic structure also has the ability to neutralize other free radicals. The quinons are reduced with quinine reductase or CYP reductase and removed by conjugation with reduced glutathione.[4]

Some authors have demonstrated that 4-OHE_2 reduces the toxic effects of oxidative stress in cells and oxidative DNA-damage.[19–21] The current study also confirm the ability of 4-OHE_2 to decrease chromium VI-induced lipid peroxidation. The metabolism of estradiol is based mainly on hydroxylation reactions induced or blocked by endo- or exogenous compounds that induce or inhibit cytochrome P450. C-4 hydroxylation leads to 4-OHE_2 formation, but C-16 hydroxylation leads to 16α-OHE_1.

The influence of estradiol and its metabolites on oxidative stress has been widely discussed in the literature, however there is little information about the role of estradiol in xenobiotic-induced oxidative stress.[22] Sowers et al. hypothesized that physiological levels of estradiol and its metabolites 2-hydroxyestrone and 16α-hydroxyestrone decrease oxidative stress measured as isoprostane levels in women's plasma; unfortunately, a test on 1647 smoking and non-smoking women at the age of 47–57 did not confirm this hypothesis.[23] On the other hand, Tang et al. showed that 4-hydroxyestrone acted as an inhibitor of lipid peroxidation in tissue cultures, while Seeger et al. demonstrated that 2-metoxyestrone and 2-hydroxyestrone blocked lipid peroxidation more efficiently than estradiol and 16α-hydroxyestrone in the serum of healthy subjects.[24,25]

Estrogens may act as pro-oxidants or antioxidants depend on their concentration.[26] The present study shows that in contrast to 4-OHE_2, 16α-OHE_1 does not decrease CrVI-induced lipid peroxidation. The advantageous effect of 4-OHE_2 on lipid peroxidation in mitochondria suggests that the increase in TBARS in mitochondria exposed to chromium treated with 17β-estradiol described in the authors' earlier publication is in fact a result of substrate not metabolite interaction between E2 and CrVI; 4-OHE_2 is not involved in this interaction.[3] The mechanism of the antioxidative action of 4-OHE_2 in exposure to CrVI seems to be connected with stimulation of SOD activity, especially in erythrocytes exposed to CrVI. While previous studies have shown that CrVI compounds strongly inhibit SOD activity, the present work demonstrates that this effect can be repaired by 4-OHE_2.[27] It can be concluded that increased formation of 4-OHE_2 in estradiol biotransformation appears beneficial for the inhibition of oxidative stress in mitochondria and erythrocytes exposed to CrVI compounds.

Human erythrocytes seem to be anappropriate model to study the estrogen-chromium effect on the antioxidative barrier. They contain antioxidative enzymes (SOD, catalase, GPx) and non-enzymatic antioxidants (vitamins C and E, beta-carotene and uric acid). Additionally, CrVI has the ability to accumulate in human erythrocytes. It has been shown that increased levels of estrogens in erythrocytes in female rats protect them from induced trauma/hemorrhagic shock (T/HS). In comparison to erythrocytes in male rats, a positive action in lowering red blood cells' deformability was observed.[28,29] The current study compared 2 cell models. Both mitochondria and erythrocytes were sensitive to the influence of chromium- and estrogen-caused oxidative disturbances. In most cell types mitochondria are the predominant source of reactive oxygen species (ROS), including superoxide anion, H_2O_2 and •OH. Although estrogen synthesis occurs in mitochondria, exogenously added estrogens are also transported to this organelle;their lipophilic properties allow them to diffuse through the lipid bilayer of cell membranes.[30] Mitochondria have a unique characteristic that allows them to participate in growth signal transduction. They are a regulatable source of ROS in response to external stimuli, for example metaloestrogens or estrogens.[31]

Mitochondria are a major target of estrogens. It appears that through their interaction with the cytoskeleton, export of cleaved signaling peptides, and/or generation of ROS, mitochondria may transduce signals to the nucleus for the activation of transcription factors involved in the cell cycle progression of estrogen-dependent cells. These interactions between estrogens and mitochondria merit future investigation, which could shed new light on the role of mitochondria in cell growth. In the present study these organelles were used to examine the interactions between estrogens and metalloestrogen in reaction to oxidative stress.[25,30,32]

Considering the results obtained in relation to CrVI-induced carcinogenesis, the 4-OHE_2 metabolite demonstrated a positive effect on the free radical reaction, while

16α-OHE_1 did not. These results support the general assumption that the introduction of another phenol group to the aromatic ring of estradiol (4-OHE2) increases the free radical scavenging properties of the metabolite-formed. The 16α-OHE_1 metabolite also shows a positive effect, but by a different mechanism. It influences the second phase of detoxification, thus increasing GST activity. It can be postulated that the biotransformation of estradiol to 4-OHE_2 and 16α-OHE_1 in exposure to CrVI has advantageous results.

Conclusions

4-hydroxyestradiol reduces the level of lipid peroxidation induced by CrVI compounds in mitochondria and in erythrocytes, increasing SOD activity. 16α-hydroxyestrone increases the activity of GST in erythrocytes exposed to CrVI.

References

1. Byrne C, Divekar SD, Storchan GB, Parodi DA, Martin MB. Metals and breast cancer. *J Mammary Gland Biol Neoplasia*. 2013;18:63–73.
2. Mishra AK, Mohanty B. Effect of sublethal hexavalent chromium exposure on the pituitary-ovarian axis of a teleost, channapunctatus (bloch). *Environ Toxicol*. 2010;27:415–422.
3. Rembacz K, Sawicka E, Długosz A. Role of estradiol in chromium–induced oxidative stress. *Acta Pol Pharm*. 2012; 69:1372–1379.
4. Licznerska B, Baer-Dubowska W. Estrogen intracrinology: Therapy and chemoprevention of brest cancer. *Post Hig Med Dosw*. 2010;64:220–230.
5. Martin BM, Reiter R, Pham T, et al. Estrogen-like activity of metals in Mcf-7 breast cancer cells. *Endocrinology*. 2003;144:2425–2436.
6. Soto AM, Calabro JM, Prechti NV, et al. Androgenic and estrogenic activity in water bodies receiving cattle feedlot effluent in eastern Nebraska USA. *Environ Health Persp*. 2004;112:346–352.
7. Matejczyk M, Zalewski P. Związki endokrynnie aktywne i ich aktywność biologiczna. *Kosmos*. 2011;60:17–32.
8. Belous AR, Hachey DL, Dawling S, Roodi N, Parl FF. Cytochrome P450 1B1-mediated estrogen metabolism results in estrogen-deoxyribonucleoside adduct formation. *Cancer Res*. 2007;67:812–817.
9. Długosz A, Sawicka E, Marchewka Z. Styrene and ethylene glycol heave a synergistic effect on lipids peroxidation, that is better protected than repaired by CoQ_{10}. *Toxicol In Vitro*. 2005;19:581–589.
10. Lowry OH, Rosbrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem*. 1951;193:265–275.
11. Długosz A, Piotrowska D. Lipid peroxidation stimulated by solvesso, bawanol and methanol and its counteraction by antioxidants in human placental mitochondria. *Toxicol In Vitro*. 2002;16:649–656.
12. Buege JA, Aust SD. Microsomal lipid peroxidation. *Methods in Enzymology*. 1978;52:302–310.
13. Stocks J, Offerman EL, Model CB, Dormandy TL. The susceptibility to autooxidation of human red cell lipids in health and disease. *Br J Haematol*. 1972;23:713–724.
14. Rice-Evans CA, Diplock AT, Symons MCR. Techniques in free radical research. Amsterdam, London, New York, Tokyo, Elsevier; 1991.
15. Mistra HP, Fridovich I. Superoxide dismutase: Photo-chemical augmention assay. *Arch Biochem Biophys*. 1985;181:308–312.
16. Woolliams JA, Wiener G, Anderson PH, McMurray CH. Variation in the activities of glutathione peroxidase and superoxide dismutase and in the concentration of copper in the blood in various breed crosses of sheep. *Res Vet Sci*. 1983;34(3):253–256.
17. Tamaki H, Yamamoto K, Kumagai H. Expression of two gluthatione S-transferase genes in the yeast issatchenkiaorientalis is induced by o-dinitrobenzene during cell growth arrest. *J Bacteriol*. 1999;181: 2958–2962.
18. Asatiani N, Sapojnikova N, Abuladze M, et al. Effects of CrVI long-term and low-dose action on mammalian antioxidant enzymes (an in vitro study). *J Inorg Biochem*.2004;98:490–496.
19. Stachowiak G, Połać I, Jędrzejczyk S, Pertyński T, Stetkiewicz T. Antyoksydacyjne działanie estrogenów. *Prz Menopauz*. 2003;2:19–22.
20. Rogan E. Xenoestrogens, biotransformation and differential risks for breast cancer. *Altern Ther Health Med*. 2007;13:112–121.
21. Muñoz-Castañeda JR, Muntané J, Muñoz MC, Bujalance I, Montilla P, Túnez I. Estradiol and catecholoestrogens protect against adriamycin-induced oxidative stress in erythrocytes of ovariectomized rats. *Toxicol Lett*. 2005;160:196–203.
22. Długosz A, Roszkowska A, Zimmer M. Oestradiol protects against the harmful effects of fluoride more by increasing thiol group levels than scavenging hydroxyl radicals. *Basic Clin Pharmacol Toxicol*. 2009;105:366–373.
23. Sowers MF, Mc Connell D, Jannausch ML, et al. Oestrogen metabolites in relation to isoprostanes as a measure of oxidative stress. *Clin Endocrinol*. 2008;68:806–813.
24. Tang M, Abplanalp W, Ayres S, Subbiah MT. Superior and distinct antioxidant effects of selected estrogen metabolites on lipid peroxidation. *Metab Clin Exp*. 1996;45:411–414.
25. Seeger H, Mueck AO, Lippert TH. Effect of estradiol metabolites on the susceptibility of low density lipoprotein to oxidation. *Life Sci*. 1997;61:865–868.
26. Świtalska M, Strządała L. Non-genomic action of estrogens. *Post Hig Med Dosw*. 2007;61:541–547.
27. Długosz A, Rembacz KP, Pruss A, Durlak M, Lembas-Bogaczyk J. Influence of chromium on the natural antioxidant barrier. *Pol J Environ Stud*. 2012;21:331–335.
28. Doucet DR, Bonitz RP, Feinman R, Colorado I, Ramanathan M, Feketeova E. Principles and practice of medicine. In: Churchill Livingstone, ed. New York, 2002:889–956.
29. Machiedo GW, Zaets S, Berezina T, Xu DZ, Spolarics Z, Deitch EA. Red blood cell damage after trauma-hemorrhage is modulated by gender. *J Trauma*. 2004;56:837–844.
30. Felty Q, Roy D. Estrogen, mitochondria and growth of cancer and non-cancer cells. *J Carcinog*. 2005;4:1–18.
31. Dugan LL, Sensi SL, Canzoniero LM, Handran SD, Rothman SM, Lin TS. Mitochondrial production of reactive oxygen species in cortical neurons following exposure to N-methyl-D-aspatrate. *J Neurosci*. 1995;15:6377–6388.
32. Kabat GC, O'Leary ES, Gammon MD, et al. Estrogen metabolism and breast cancer. *Epidemiology*. 2006;17:80–88.

Collagenous scaffolds supplemented with hyaluronic acid and chondroitin sulfate used for wound fibroblast and embryonic nerve cell culture

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D – writing the article; E – critical revision of the article; F – final approval of article

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Conflict of interest
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Abstract

Background. Tissue engineering is a strategy aimed at improving the regeneration of injured tissues.

Objectives. The aim of the present study was to determine whether a tri-copolymer composed of cross-linked collagen, chondroitin sulfate and hyaluronic acid (Col + CS + HA) provides a better environment for fibroblast and embryonic nerve cell culture than a collagenous scaffold (Col).

Material and methods. The porosity of each of the matrices was characterized with a scanning electron microscope. Fibroblasts were isolated from rat wound granulation tissue (polypropylene net implanted subcutaneously). Embryonic nerve cells were obtained from the brains of rat embryos. The cells were applied to scaffolds and then stained with bisbenzimide to calculate cell entrapment within the material. The metabolic activity of the cells cultured within the scaffolds was tested using the 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

Results. The Col scaffolds had a homogenously porous structure with a pore diameter of 50 µm for 70% of pores. The pore diameter in the tri-copolymer (Col + HA + CS) ranged from 24 to 160 µm (95% of total pore volume). Four times more cells (fibroblasts and embryonic nerve cells) were trapped within the superficial part of the collagenous scaffold than that of the tri-copolymer. On the third day of culture the metabolic activity of the fibroblasts within the 2 tested scaffolds was significantly higher than in the control conditions (cell culture on a laminin-coated surface). Also, the embryonic nerve cells demonstrated increased metabolic activity in Col + CS + HA scaffolds than the Col scaffolds.

Conclusions. Both fibroblasts and embryonic nerve cells could be seeded within the 2 tested scaffolds. Both the scaffolds provide good conditions for fibroblast culture. However, the Col + CS + HA tri-copolymer is preferable for embryonic nerve cell engineering.

Key words: collagen, fibroblasts in vitro, embryonic nerve cells

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Large wounds lead to disturbances of homeostasis. Immediately following an injury, hemostasis and inflammation are induced. These processes are followed by cell migration and proliferation phases, after which fibroblasts begin the synthesis of an extracellular matrix. Together, these processes lead to scar formation that rearranges the continuity of the tissue.[1] Large wounds heal very slowly due to the large space that needs to be covered by the scar. Although limited results can be obtained by treating large wounds with conventional methods, tissue engineering provides a chance to achieve better outcomes.

Cell engineering is a new strategy developed to regenerate nerve tissues. The engineered cells are supported by a biodegradable scaffold that not only forms a mechanical strut, but also may exert a regulatory influence on cell migration, proliferation and extracellular matrix synthesis. Thus, the implants obtained not only close the wound but also stimulate the self-repair or self-regeneration of the wound.[2]

The favorable physical and chemical properties of collagenous (Col) scaffolds for regenerative medicine have been described by Pietrucha et al.[3–6] The Col materials create a supportive microenvironment for fibroblasts, osteoblasts and embryonic nerve cells.[2,6–8] Wound fibroblasts are thought to enter the scaffold from the margins of the wound and participate in scar formation. Collagen may regulate the function of cells in the wound by binding to the integrin subfamily (α1β2, α2β2, α10β2 and α11β2), which would influence granulation tissue remodeling, angiogenesis, fibroblast adhesion and myofibroblast differentiation in the wound.[9]

Hyaluronic acid (HA) is a polysaccharide composed of glucuronic acid and N-acetylglucosamine. A scaffold containing hyaluronic acid promotes wound repair in rats, accelerates wound closure, supports re-epithelialization and angiogenesis in mice, and stimulates fibroblasts to release growth factors in rats.[10–11] Chondroitin sulfate (CS), composed of glucuronic acid and N-acetylgalactosamine, was found to promote fibrotic scar formation in an injury of the central nervous system.[12] CS is negatively charged due to the presence of SO_4^{2-} and COO^- moieties in the molecule, and this negative polarity allows the adhesion of growth factors and cells. In addition, CS influences the migration of cells.[13]

Some studies report that scaffolds composed of collagen, HA and CS have beneficial effects when used for skin or cartilage regeneration.[14,15] The materials were seeded with skin fibroblasts or adipose-derived stem cells.[14] Li et al. constructed a scaffold composed of collagen, hyaluronic acid and chondroitin-6-sulfate for nucleus pulposus tissue engineering. The copolymer was characterized by a highly porous structure, hydrophilic properties, good mechanical stability, low immunogenicity and good biocompatibility.[16]

The aim of the present study was to determine whether supplementation of a Col scaffold with CS and HA can influence the biological properties necessary for fibroblast and embryonic nerve cell culture, so that the scaffold providing the best environment for a given purpose can be selected. A previous study found that a scaffold composed of collagen cross-linked with CS constitutes a good environment for embryonic nerve cells.[6] A novel element of the present study was that it investigated whether a collagen tri-copolymer with the addition of HA and CS (Col + HA + CS) can enhance cultures not only of embryonic nerve cells, but also wound fibroblasts. In this way, the study determined the usefulness of this tri-copolymer for tissue engineering.

Material and methods

The animals

The experiments were performed on 7 male and 9 female Wistar rats weighing 230 ± 40 g. The animals were kept on a 12 : 12 light/dark cycle (from 7:00) and were given free access to standard rat feed (Bakutil, Radzyń Podlaski, Poland) and tap water ad libitum. The study was approved by the Local Ethics Committee in Łódź, Poland.

Wound model

A 4 cm skin incision was made in the left lumbar region of each anesthetized rat and 3 × 2 cm polypropylene mesh was inserted subcutaneously. The incision was closed with 4 silk sutures. Four weeks after the wound was made, the implant, overgrown by connective tissue, was removed under aseptic conditions.[17]

Fibroblast isolation and culture

The removed implants containing granulation tissue were stored in RPMI 1640 medium (Gibco, Paisley, UK) containing gentamicin (25 μg/mL) and fungizone (2.5 μg/mL). After mincing, the tissue was incubated in 0.1% collagenase solution (37°C, 5% CO_2) for 40 min. After centrifugation (5 min, 1000 rpm), the samples pellets were washed with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, gentamicin and fungizone (Gibco, Paisley, UK), then centrifuged and plated on dishes. The non-adherent cells were washed out after 2 h. The cells were cultured in a CO_2 incubator at 37°C in a 100% humidified atmosphere of 5% CO_2 and 95% air. Six-well plates (TPP Techno Plastic Products AG, Trasadingen, Switzerland) were used for cell culture.

After reaching confluence, the cells were trypsinized and passaged to new flasks. New cultures were set up at an initial cell density of $8 \times 10^3/cm^2$. Bürker's chamber was used for cell counting. Necrotic cells were stained using trypan blue elimination assays. After the second passage, the experiments were carried out in DMEM contain-

ing 3% calf serum and antibiotics at the concentrations given above; the calf serum concentration was lowered to decrease interference from fibroblast-scaffold interactions. The isolated cells were identified as fibroblasts as described in an earlier study by the current authors.[17] Both fibroblasts and embryonic nerve cells were used in 2 experiments for cell entrapment and for 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays. Each experimental group consisted of 7 wells with the cells of different animals.

Isolation and culture of embryonic nerve tissue

Pregnant Wistar rats were euthanized and embryos at day 17 of gestation were isolated from the uterus in cold phosphate buffered saline (PBS, Gibco, Paisley, UK). After removal of the brains, the meninges and blood vessels were excised. The brain samples were incubated with 1 mL of enzymatic solution composed of collagenase (1 mg/mL), (Sigma, St. Louis, USA) and dispase (2 mg/mL), (Gibco, Paisley, UK) at room temperature (5 min) to remove the connective tissue. The brains were then incubated with 2 mL trypsin (5 min at 37°C), (Gibco, Paisley, UK). The trypsin was neutralized with 5 mL of medium, (MACS NeuroBrew-21, Miltenyi Biotec, Bergisch Gladbach, Germany) after which the sample was centrifuged at 1000 rpm.

The cells were resuspended with 100 µL of medium (MACS NeuroBrew-21, Miltenyi Biotec, Bergisch Gladbach, Germany) and gently triturated (3-5 X). The cells were then seeded on laminin-coated 6-well plates (TPP, Trasadingen, Switzerland).

The embryonic nerve cells were cultured in MACS NeuroBrew-21 containing gentamicin (25 µg/mL) and fungizone (2.5 µg/mL). The phenotype of the cells was determined by flow cytometry. The entrapment study and MTT experiments were performed on cultures of cells derived from 9 animals.

Flow cytometry experiments

Flow cytometry experiments were performed to determine the phenotype of the isolated embryonic nerve cells. After fixation with BD Cytofix (BD Biosciences, Franklin Lakes, USA), the embryonic nerve cells were permeabilized by incubation with BD Phosflow Perm Buffer III (BD Biosciences, Franklin Lakes, USA). The samples were then washed with PBS and centrifuged (5 min, 1000 rpm).

The permeabilized cells were stained (4–8°C for 30 min) in BD Pharmingen stain buffer (BD Biosciences, Franklin Lakes, USA), with the following antibodies:

– mouse anti-Microtubule-Associated Protein 2B (MAP2B) conjugated with Alexa Fluor 488 (BD Biosciences, Franklin Lakes, USA);
– mouse anti-Myelin Basic Protein (MBP) conjugated with PE (Abcam, Cambridge, USA);
– mouse anti-Glial Fibrillary Acidic Protein (GFAP) conjugated with PE (BD Pharmingen, San Diego, USA);
– goat anti-mouse IgG2b heavy chain isotype control conjugated with FITC (BD Pharmingen, San Diego, USA);
– mouse IgG2b isotype control conjugated with PE (BD Pharmingen, San Diego, USA);
– mouse IgG1 k isotype control conjugated with Alexa Fluor 488 (BD Pharmingen, San Diego, USA).

The stained cells (3×10^4/samples) were analyzed using a Becton-Dickinson FACScan analytical flow cytometer (Becton Dickinson, Franklin Lakes, USA).

Preparation and characterization of ternary composite collagen-based scaffolds

The collagen/hyaluronic acid/chondroitin sulfate (Col + + HA + CS) matrices were constructed as described previously, with some modifications.[18] In brief, a dispersion in dilute acetic acid (pH 3.2) of type I porcine collagen (Col) (Euroimplant, Rybie, Poland), hyaluronic acid (HA) from *Streptococcus equi* with molecular weight of 1.6×10^3 kDa, (Sigma-Aldrich, St. Louis, USA) and chondroitin sulfate (CS) from shark cartilage with an average molecular weight of 20 kDa, (Sigma-Aldrich, St. Louis, USA) at a ratio of 6 : 1 : 1 was freeze-dried at –50°C to form 3D sponge-shaped porous scaffolds. For comparison, sponges from pure Col were also prepared. To improve the functional properties of the scaffolds, all the collagenous sponges were treated with a carbodiimide-based process as previously described, but with significant modifications: The sponges were cross-linked at room temperature by immersion in an 80% ethanol-water solution containing 33 mM 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC) and 6 mM N-hydroxysuccinimide (NHS).[19,20] After the reaction, the matrices were thoroughly washed in 0.1 M Na_2HPO_4, and then with deionized water. Finally, the cross-linked matrices were again lyophilized at –55°C. All the collagen-based scaffolds were sterilized with ethylene oxide (EtO). The multi-component porous sponges were then applied as matrices for culturing cells to form a hybrid tissue-like structure intended for cell engineering.

As noted above, the addition of hyaluronic acid to an earlier investigated scaffold (Col + CS) created a tri-copolymer (Col + CS + HA), the potential of which for embryonic nerve cell and wound fibroblast culture had not been investigated prior to the present study.[6]

The porosity of each of the matrices was characterized using a Nova NanoSEM 230 scanning electron microscope (FEI Company, Eindhoven, the Netherlands) with an Apollo 40 SDDEDS X-ray microanalyser (EDAX McKee Drive Mahwah, USA). The operating parameters were as follows: A low vacuum setting of 70 Pa, an accelerating voltage of 10 kV and LVD detector selected. Cross-sections or surface sections of the sponge were obtained

by breaking the specimens after freezing them in liquid nitrogen. An image analysis application was used to determine the average diameter of the pores, with at least 200 pores assessed on each specimen at ×100 and ×400 magnification. Fig. 1 shows the results of the pore analysis of 2 collagen-based scaffolds.

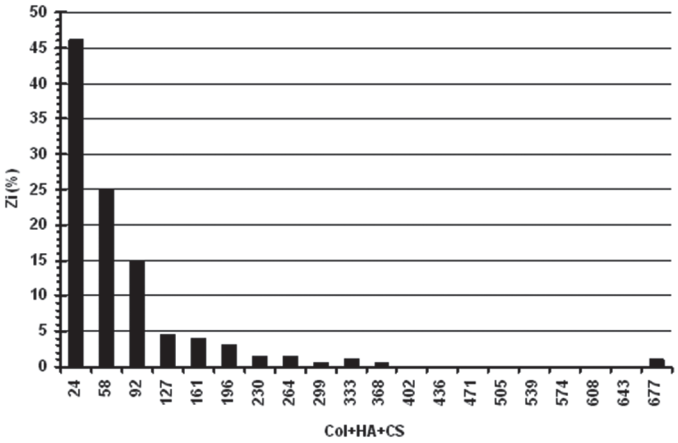

Fig. 1. Distribution of pore diameters of collagen-based scaffolds modified with EDC and NHS: (K1) scaffold composed of collagen

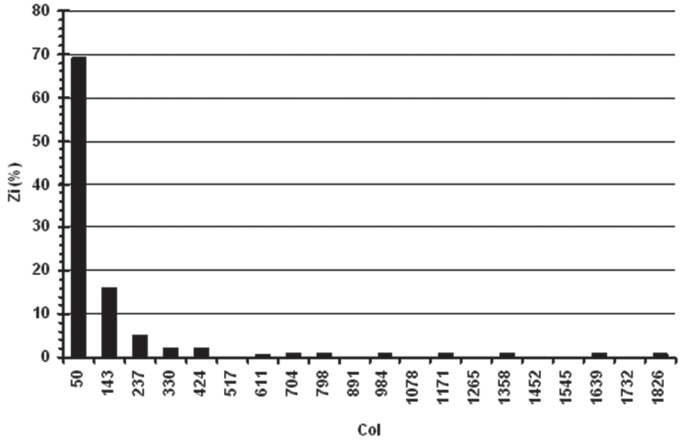

Fig. 2. Distribution of pore diameters of collagen-based scaffolds modified with EDC and NHS: (K2) tri-copolymer scaffold composed of collagen, chondroitin sulfate and hyaluronic acid

Cell entrapment study

Wound fibroblasts were seeded on the scaffolds at a density of 10^4/well of 96-well plates (TPP, Trasadingen, Switzerland) and incubated for 24 h in DMEM containing 3% fetal calf serum, gentamicin and fungizone (37°C, 5% CO_2). Embryonic nerve cells were added to a 96-well plate at a density of 10^4 cells per well. They were then incubated for 24 h in MACS NeuroBrew-21 medium (Miltenyi Biotec, Bergisch Gladbach, Germany). After being washed with PBS, the samples were incubated for 30 min (37°C, 5% CO_2) with bisbenzimide (1 µg/mL in PBS). After incubation, the scaffolds were washed with PBS, and the stained nuclei of the fibroblasts were counted under the microscope at ×100 magnification.[3]

MTT experiments

The experiments were performed in 96-well plates (TPP, Trasadingen, Switzerland). The cells were applied to the scaffold at a density of 10^4 cells per well. After 2 or 3 days of culture, the samples were washed 3 times with PBS, and then 50 µL of 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT reagent, 5 mg/mL, Sigma, St. Louis, USA) was added to each well. The samples were then incubated for 4 h at 37°C in a humid atmosphere containing 5% CO_2. The MTT solution was removed and 200 µL DMSO was added to each sample and incubated for 10 min. After the addition of Sorensen's buffer (0.1 M NaCl + 0.1M $C_2H_5NO_2$, pH = 10.5), the absorbance was determined at a wavelength of 570 nm using an EL × 800UV Universal Microplate Reader (BioTek Instruments Inc., Winooski, USA). Three groups of cells were studied: control cultures (2D), cultures on Col scaffolds and cultures on Col + CS + HA scaffolds. The control cells were seeded on a laminin-coated surface.

Statistical analysis

The Kruskal-Wallis test was used for the statistical analysis. Statistical differences between more than 2 independent groups were evaluated by multiple comparisons of mean ranks. The U-Mann Whitney test was used for the evaluation of statistical differences between 2 independent groups. The statistical differences between 2 dependent groups were calculated using the t-test for 2 dependent samples (when the data distribution was normal) or the Wilcoxon test (when the data distribution was not normal). The threshold of significance was $p < 0.05$. The calculations were performed using STATISTICA 12.5 software (Dell Statistica, Tulsa, USA).

Results

The Col scaffolds were found to have a homogenously porous structure with a pore diameter of 50 µm in 70% of the pores. For 15% of the total pore volume, the pore diameter ranged from 50 µm to 140 µm. However, for the tri-copolymer (Col + HA + CS), the pore diameter ranged from 24 to 160 µm for 95% of the total pore volume of the scaffold (Figs. 1 and 2).

Fibroblasts were isolated from the granulation tissue of the wound. The phenotype of the cells was confirmed by electron microscopy as described in an earlier study.[17] The embryonic nerve cells were differentiated into neurons (MAP2B positive cells, Fig. 3), oligodendrocytes (MBP positive cells, Fig. 4) and astrocytes (GFAP positive cells, data not shown).[6]

Fibroblasts were able to enter both Col and Col + CS + + HA scaffolds, but the Col + CS + HA scaffolds were less populated on the upper surface. Statistically signifi-

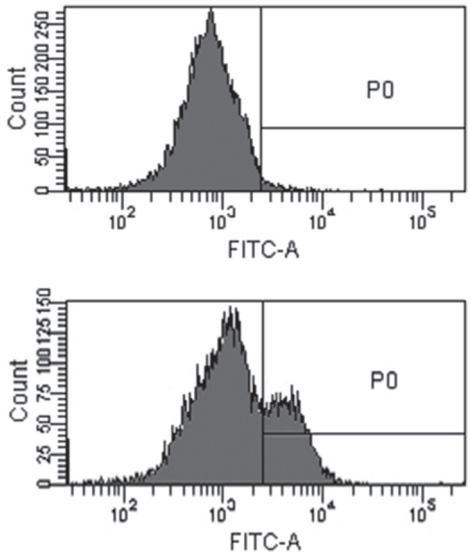

Fig. 3. Fluorescence-activated cell sorting (FACS) profiles showing the expression of microtubule-associated protein (MAP2b) on embryonic nerve cells (lower panel) compared with the control (upper panel)

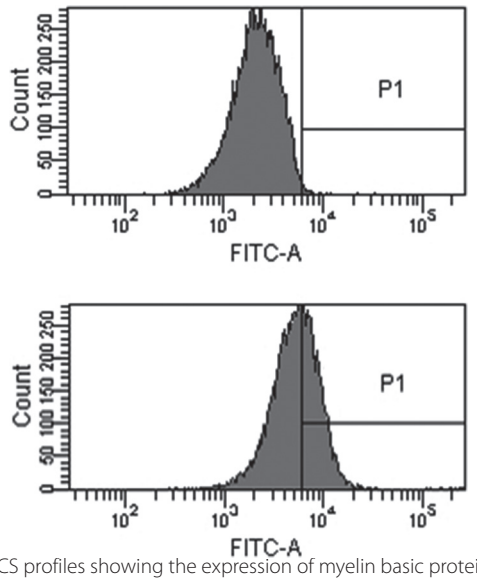

Fig. 4. FACS profiles showing the expression of myelin basic protein (MBP) on embryonic nerve cells (lower panel) compared with the control (upper panel)

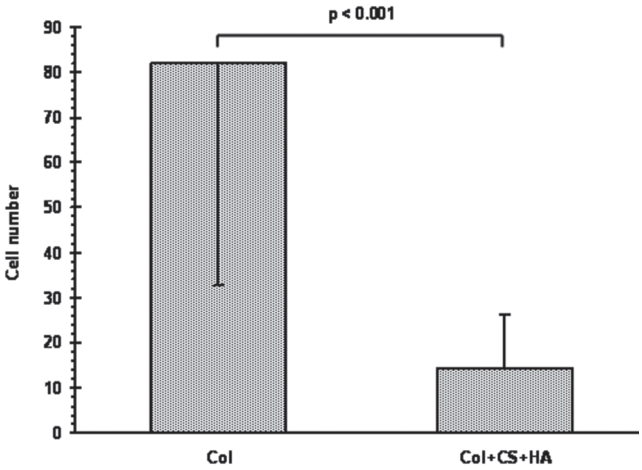

Fig. 5. Number of fibroblasts stained with bisbenzimide entrapped by scaffolds made of collagen (Col) and of collagen with chondroitin sulfate and hyaluronic acid (Col + CS + HA). Each value expresses the mean of 6 samples ± standard deviation (SD)

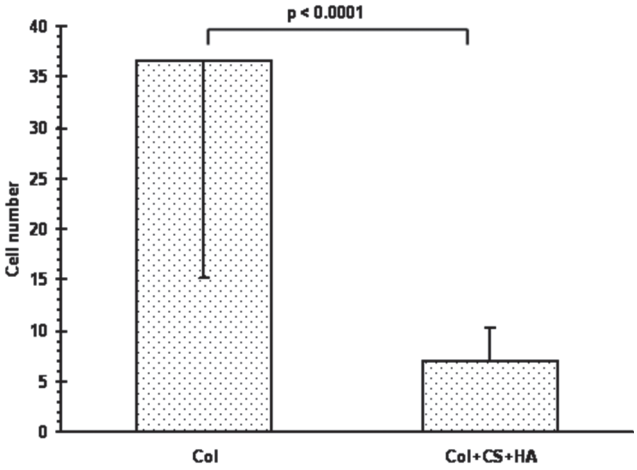

Fig. 6. Number of embryonic nerve cells stained with bisbenzimide entrapped by scaffolds made of collagen (Col) and of collagen with chondroitin sulfate and hyaluronic acid (Col + CS + HA). Each value expresses the mean of 6 samples ± standard deviation (SD)

cant differences were observed in the numbers of fibroblasts infiltrating the Col and Col + CS + HA scaffolds (Fig. 5). At the same time, the embryonic nerve cells were entrapped by both the Col and Col + CS + HA scaffolds, but significantly higher numbers of cells were found in the former than the latter (Fig. 6).

The metabolic activity of the cells was analyzed after 2 or 3 days of culture. On the 2nd day, lower fibroblast metabolic activity was found in the Col + CS + HA scaffolds than in the controls (a 2D cell culture on a laminin-coated surface). On the 3rd day, however, the metabolic activity of the fibroblasts in Col and Col + CS + HA scaffolds was higher than in the controls. Interestingly, while the metabolic activity of the fibroblasts fell from day 2 to day 3 in the control cultures, the opposite occurred in the scaffolds composed of Col + CS + HA, with fibroblast activity doubling from day 2 to day 3 (Fig. 7).

On the 2nd and 3rd days of culture, embryonic nerve cells within the Col scaffolds demonstrated decreased metabolic activity compared with the controls. In the Col scaffolds, the metabolic activity of the embryonic nerve cells was significantly higher on the 3rd day of culture than on the 2nd day. Within the Col + CS + HA scaffolds, the embryonic nerve cells demonstrated increased metabolic activity on both days 2 and 3 of culture compared to the Col scaffolds (Fig. 8). Lower metabolic activity of both fibroblasts (Fig. 7) and embryonic nerve cells (Fig. 8) was noted on day 3 of culture, compared with day 2 in the control groups.

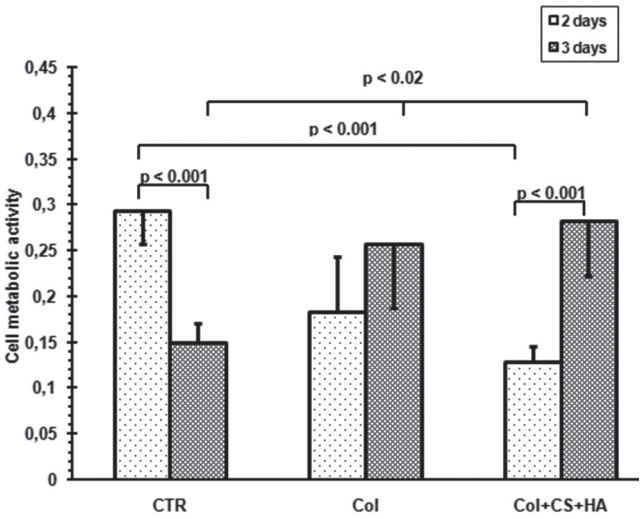

Fig. 7. Metabolic activity of fibroblasts cultured for 2 or 3 days on a laminin-coated surface (CTR), in collagen scaffolds (Col) and in scaffolds composed of collagen, chondroitin sulfate and hyaluronic acid (Col + CS + HA). Each value expresses the mean of 8 samples ± standard deviation (SD)

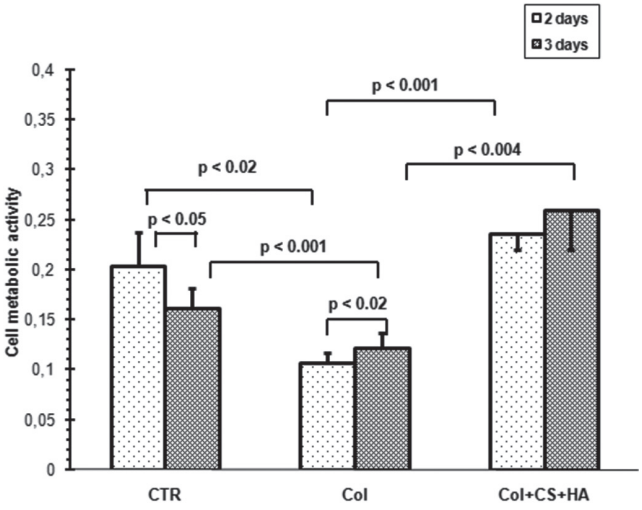

Fig. 8. Metabolic activity of embryonic nerve cells cultured for 2 or 3 days on a laminin coated surface (CTR), in collagen scaffolds (Col) and in scaffolds composed of collagen chondroitin sulfate and hyaluronic acid (Col + CS + HA). Each value expresses the mean of 8 samples ± standard deviation (SD)

Discussion

Earlier studies indicated that the secondary triple helix structure of collagen present within Col scaffolds remained unchanged, thus allowing the optimal biological properties of the scaffold to be used in regenerative medicine.[3–6] In the present study, the presence or absence of cross-links, CS and HA in the collagen sponges had a significant effect on the overall structure and distribution of the interconnected pores (Figs. 1, 2). After cross-linking collagen alone, the sponges exhibited an almost homogenous distribution of pores, with 70% of the pores having a diameter of 50 μm (Fig. 1, K1). Moreover, the results also showed that pore sizes ranging from 50 to 140 μm made up about 15% of the total pore volume. Conversely, the pore size of the ternary composite Col-HA-CS sponges, cross-linked with EDC/NHS, was significantly lower than in the collagen sponges without HA and CS (Fig. 2, K2). The largest fraction, comprising at least 70% of the total pore volume of the sponge, was occupied by pores ranging from 24 to 90 μm in size. Only 15% of the Col + HA + CS scaffolds contained pores much larger than 90 μm, i.e. ranging between 127 and 196 μm. Wang et al. reported that the pores of tri-copolymer Col + CS + HA (9 : 1 : 1) scaffolds were uniform and widely interconnected, with a mean diameter of 109 μm.[21] The dimensions of pores in collagenous scaffolds prepared by Yannas et al. ranged from 20 to 125 μm.[22] Huang et al. observed that in a collagen and glycosaminoglycan copolymer, the pore size ranged from 20 to 140 μm.[23]

The present study showed good fibroblast and embryonic nerve cell entrapment within the Col scaffolds (Figs. 5, 6). The presence of a relatively high density of cells settled within the Col material implies appropriate porosity, allowing the passage of different cell types into the scaffold. Moreover, more fibroblasts than embryonic nerve cells were entrapped in the Col scaffold. This effect is believed to be caused by the size and shape of fibroblasts, which may be a better fit for the pores. In addition, the Col + CS + HA scaffolds were about 4 times less populated than the Col scaffolds and controls. Thus, supplementation of the scaffold with chondroitin sulfate and hyaluronic acid decreases the ability of the Col scaffold to catch both fibroblasts and embryonic nerve cells. In a previous study, the numbers of embryonic nerve cells in the Col scaffolds and in a Col-chondroitin sulfate composite were found to be the same.[6] In the present study, the addition of HA may disrupt the closed, honeycomb-like structure of the Col scaffold and cause the formation of open interconnections between pores, leading to the low populations of seeded fibroblasts and embryonic nerve cells observed in the tri-copolymer Col + CS + HA scaffolds.[24] Although all the applied cells were entrapped within the scaffolds, different numbers of cells were found on the superficial parts of the scaffolds.

The metabolic activity of fibroblasts and embryonic nerve cells in the control groups (Figs. 6, 7) was found to be lower on the 3rd day of culture than on the 2nd day. This effect was probably caused by the limitations of a 2D culture and intensive cell-cell interactions. Bracke et al. reported inhibition of several energy-requiring activities in confluent epithelial cells, but MTT conversion increased only in confluent cells with a dysfunctional E-cadherin/-catenin complex, implying that cell metabolism may be regulated by cell-cell adhesion via the E cadherin/catenin complex.[25] Interestingly, this effect was not observed in 3D cultures created within the scaffolds (Figs. 7, 8). Thus, both Col and Col + CS + HA scaffolds provide favorable environments for fibroblast culture.

The results of the present study confirm that the metabolic activity of fibroblasts within the 2 types of scaffold were better on the 3rd day than the 2nd day of the culture

(Fig. 7). In addition, on the 2nd day of culture, the fibroblasts within the Col + CS + HA scaffolds demonstrated lower metabolic activity than in the controls. However, a rapid increase in metabolic activity was seen in the Col + CS + HA scaffolds on the 3rd day of culture. These findings confirm those of earlier studies showing that a collagen-containing scaffold has good properties for tissue engineering.[2,6,7] Fibroblasts seeded in collagen-gelatin sponges have been used as a drug delivery system, as they allow up-regulation of growth factor synthesis; these sponges appeared to be biocompatible with fibroblasts.[26] Moreover, fibroblasts cultured within scaffolds with unidirectional collagen fibers secrete regulatory factors that modulate the wound-healing process.[27] The collagen present in the extracellular space may also influence endogenous collagen expression.[28]

In the present study, the Col scaffolds did not guarantee good conditions for the culture of embryonic nerve cells: The cells seeded within the Col material displayed lower metabolic activity than in either the control cultures or the Col + CS + HA scaffolds. These results correspond with the data from the authors' previous study showing that embryonic nerve cells cultured within a scaffold composed of collagen and chondroitin sulfate demonstrate better metabolic activity than those in a Col scaffold.[6] The authors therefore recommend the tri-copolymer for embryonic nerve cell engineering.

The authors' earlier study showed that a Col scaffold supplemented with CS provides good conditions for embryonic nerve cell culture.[6] The addition of CS improved the metabolic activity of the cells compared with those in the Col scaffold.[6] The present study found that cells seeded in tri-copolymer scaffolds demonstrated better metabolic activity than those in scaffolds composed of Col alone. This reaction is believed to be due to hyaluronic acid modifying the structure of collagen, allowing the formation of interconnections between pores, which supports the migration of cells to internal pores, allows for better communication between cells and improves the nutrition of the seeded cells.[24] Since high concentrations of hyaluronic acid, chondroitin sulfate and heparin sulfate are known to be present in the brain, the collagenous scaffold was supplemented with glycosaminoglycans for nerve cell culture.[29] Chondroitin sulfate is known to exert a regulatory influence on the differentiation and migration of endogenous neural precursor cells.[23] In addition, stem cells seeded in scaffolds containing hyaluronic acid were characterized with better survival and differentiation.[30]

Conclusions

The study showed that fibroblasts and embryonic nerve cells were entrapped by both of the scaffolds used (Col and Col + CS + HA). However, cell entrapment was about 4 times more efficient within the Col scaffolds than the Col + CS + HA scaffolds for both of the tested cell cultures. Both the Col and Col + CS + HA scaffolds provided a good environment for fibroblasts; but since the scaffolds composed of Col + CS + HA guaranteed better conditions for the culture of embryonic nerve cells than Col alone, the tri-copolymer Col + CS + HA is recommended for the engineering of embryonic nerve cells.

References

1. Drobnik J. Wound healing and the effect of pineal gland and melatonin. *J Exp Integr Med*. 2012;2:3–14.
2. Guo Y, Zeng QCh, Zhang ChQ, et al. Extracellular matrix of mechanically streched cardiac fibroblasts improves viability and metabolic activity of ventricular cells. *Int J Med Sci*. 2013;10:1837–1845.
3. Pietrucha K. Physicochemical properties of 3D collagen-CS scaffolds for potential use in neural tissue engineering. *Int J Biol Macrom*. 2015;80:732–739.
4. Pietrucha K, Safandowska M. Dialdehyde cellulose-crosslinked collagen and its physicochemical properties. *Process Biochem*. 2015;50:2105–2111.
5. Pietrucha K. Changes in denaturation and rheological properties of collagen-hyaluronic acid scaffolds as a result of temperature dependencies. *Int J Biol Macrom*. 2005;36:299–304.
6. Pietrucha K, Szymański J, Drobnik J. The behavior of embryonic neural cells within the 3D micro-structured collagen-based scaffolds. *International Federation for Medical and Biological Engineering (IFMBE) Proceedings*. 2015;45:549–552.
7. Masci VL, Taddei AR, Gambellini G, Giorgi F, Fausto AM. Ultrastructural investigation on fibroblasts interaction with collagen scaffold. *J Biomed Mater Res Part A*. [published online September 16, 2015], doi: 10.1002/jbm.a.35563.
8. Quinlan E, Thompson EM, Matsiko A, O'Brien FJ, López-Noriega A. Functionalization of a collagen-hydroxyapatite scaffold with osteostatin to facilitate enhanced bone regeneration. *Adv Healthc Mater*. [published online September 28, 2015]. doi: 10.1002/adhm.201500439.
9. Koivisto L, Heino J, Hakkinen L, Larjava H. Integrins in wound healing. *Adv Wound Care (New Rochelle)*. 2014;3(12):762–783.
10. Cerqueira MT, da Silva LP, Santos TC, et al. Human skin cell fractions fail to self-organize within a gellan gum/hyaluronic acid matrix but positively influence early wound healing. *Tissue Eng Part A*. 2014;20:1369–1378.
11. Kuroyanagi M, Yamamoto A, Shimizu N, et al. Development of anti-adhesive spongy sheet composed of hyaluronic acid and collagen containing epidermal growth factor. *J Biomater Sci Polym Ed*. 2014;25:1253–1265.
12. Li HP, Homma A, Sango K, Kawamura K, Raisman G, Kawano H. Regeneration of nigrostriatal dopaminergic axons by degradation of chondroitin sulfate is accompanied by eliminationof the fibrotic scar and glia limitans in the lesion site. *J Neurosci Res*. 2007;85:536–547.
13. Zou XH, Jiang YZ, Zhang GR, Jin HM, Nguyen TM, Ouyang HW. Specific interactions between human fibroblasts and particular chondroitin sulfate molecules for wound healing. *Acta Biomater*. 2009;5:1588–1595.
14. Dinescu S, Gălăţeanu B, Albu M, et al. Biocompatibility assessment of novel collagen-sericin scaffolds improved with hyaluronic acid and chondroitin sulfate for cartilage regeneration. *Biomed Res Int*. [published online November 7, 2013]. doi: 10.1155/2013/598056.
15. Murphy CM, Matsiko A, Haugh MG, Gleeson JP, O'Brien FJ. Mesenchymal stem cells fate is regulated by composition and mechanical properties of collagen-glycosaminoglycans scaffold. *J Mech Behav Biomed Mater*. 2012;11:53–62.
16. Li CQ, Huang B, Luo G, Zhang CZ, Zhuang Y ZO, Hou Y. Construction of collagen II/hyaluronate/chondroitin-6-sulfate tri-copolymer scaffold for nucleus pulposus tissue engineering and preliminary analysis of its physic-chemical properties and biocompatibility. *J Mater Sci Mater Med*. 2010;21:741–751.
17. Drobnik J, Owczarek K, Piera L, et al. Melatonin-induced augmentation of collagen deposition in cultures of fibroblasts and myofibroblasts is blocked by luzindole – melatonin membrane receptors inhibitor. *Pharmacol Rep*. 2013;65(3):642–649.

18. Pietrucha K. Building up and characterization of multi-component collagen based scaffolds. *International Federation for Medical and Biological Engineering (IFMBE) Proceedings*. 2014;41:1626–1629.
19. Pieper JS, Oosterhof A, Dijkstra PJ, Veerkamp JH, van Kuppevelt TH. Preparation and characterization of porous collagenous matrices containing bioavailable chondroitin sulfate. *Biomaterials*. 1999;20:847–858.
20. Harley BA, Leung JH, Silva ECCM, Gibson LJ. Mechanical characterization of collagen-glycosaminoglycan scaffolds. *Acta Biomater*. 2007;3:463–474.
21. Wang W, Zhang M, Lu W, et al. Cross-linked collagen-chondroitin sulphate-hyaluronic acid imitating extracellular matrix as scaffold for dermal tissue engineering. *Tissue Eng Part C*. 2010;16:269–279.
22. Yannas JV. Emerging rules for inducing organ regeneration. *Biomaterials*. 2013;34: 321–330.
23. Huang K.F, Hsu WCH, Chiu WT, Wang JY. Functional improvement and neurogenesis after collagen-GAG matrix implantation into surgical brain trauma. *Biomaterials*. 2012;33:2067–2075.
24. Tang S, Vickers SM, Hsu HP, Spector M. Fabrication and characterization of porous hyaluronic acid-collagen composite scaffolds. *J Biomed Mater Res*. 2007;82A:323–335.
25. Bracke ME, Depypere H, Labit C, et al. Functional downregulation of the E-cadherin/catenin complex leads to loss of contact inhibition of motility and of mitochondrial activity, but not of growth in confluent epithelial cell cultures. *Eur J Cell Biol*. 1997;74:342–349.
26. Hiwatashi N, Hirano S, Mizuta M, et al. Biocompatibility and efficacy of collagen/gelatin sponge scaffold with sustained release of basic fibroblast growth factor on vocal fold fibroblasts in 3-dimensional culture. *Ann Otol Rhinol Laryngol*. 2015;124(2):116–125.
27. Bustos RH, Suesca E, Millán D, González JM, Fontanilla MR. Real-time quantification of proteins secreted by artificial connective tissue made from uni- or multidirectional collagen I scaffolds and oral mucosa fibroblasts. *Anal Chem*. 2014;86(5):2421–2428.
28. Kuzan A, Smulczyńska-Demel A, Chwiłkowska A, Saczko J, Frydrychowski A, Dominiak M. An estimation of the biological properties of fish collagen in an experimental in vitro study. *Adv Clin Exp Med*. 2015;24:385–392.
29. Rauch U. Extracellular matrix components associated with remodeling processes in brain. *Cell Mol Life Sci*. 2004;61:2031–2045.
30. Ballios BG, Cooke MJ, van der Kooy D, Shoichet MS. A hydrogel-based stem cell delivery system to treat retinal degenerative diseases. *Biomaterials*. 2010;31:2555–2564.

A comparative study of sTREM-1, IL-6 and IL-13 concentration in bronchoalveolar lavage fluid in asthma and COPD: A preliminary study

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Abstract

Background. Soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) is a cell-surface receptor presented on neutrophils, macrophages and monocytes. Elevated sTREM-1 levels are a marker of acute microbial infections, and have also been reported in chronic lung diseases. IL-6 and IL-13 are effective markers in distinguishing these diseases. IL-6 has been shown to play an important role in chronic obstructive pulmonary disease (COPD), while IL-13 is described as one of the key mediators of allergic asthma.

Objectives. The aim of the study was to evaluate the level of sTREM-1 in bronchoalveolar lavage fluid (BALF) from stable mild-to-moderate asthma and COPD patients and to compare the utility of BALF sTREM-1 measurements in asthma/COPD differentiation with those of IL-6 and IL-13.

Material and methods. The concentration of sTREM-1, IL-6 and IL-13 was evaluated by ELISA in the BALF of stable mild-to-moderate asthma and COPD patients.

Results. There were no significant differences in BALF sTREM-1 levels between asthma and COPD patients (52.5 pg/mL for asthma vs 73.4 pg/mL for COPD, $p = 0.492$), in contrast to the differences in the IL6/IL13 ratio (0.68 in COPD, 0.22 in asthma, $p = 0.043$).

Conclusions. The study showed that BALF sTREM-1 levels do not discriminate between mild-to-moderate asthma and COPD. In contrast, the IL-6/IL-13 ratio measured in BALF effectively differentiated these two diseases in their stable state. These results suggest that the most important marker of inflammation in mild-to-moderate obstructive lung disease is not microbiology-dependent.

Key words: IL-6, asthma, COPD, IL-13, sTREM-1

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Triggering receptor expressed on myeloid cells (TREM-1) is a cell-surface receptor presented on neutrophils, macrophages and monocytes. There is evidence that TREM-1 is involved in host immune response to microbial infection.[1] However, little is known about the natural ligands for TREM-1. Recently, peptidoglycan recognition protein 1 (PGLYRP1), a neutrophil granule protein with antibacterial properties, was found to be a functional ligand for TREM-1.[2] Activation of TREM-1 causes rapid neutrophil degranulation, increasing pro-inflammatory cytokine production (CXCL8, tumor necrosis factor α [TNF-α], IL-1β) and inhibiting IL-10 expression in monocytes and macrophages.[3,4] Upregulation of TREM-1 is associated with the release of its soluble form (sTREM-1), which can be detected in various biological fluids, including serum, sputum and bronchoalveolar lavage fluid (BALF).[5,6] Soluble TREM-1 is generated through proteolytic cleavage of membrane-anchored TREM-1 by matrix metalloproteinases with the subsequent release of the TREM-1 ectodomain into the microenvironment.[7] Alternatively, sTREM-1 may be secreted by neutrophils in response to lipopolysaccharide (LPS) stimulation during de novo protein synthesis.[8] Soluble TREM-1 acts as a decoy for the natural TREM-1 ligand and reduces its activation.[9] Soluble TREM-1 is detectable in infectious diseases caused by extracellular microorganisms. Earlier studies have shown that an elevated level of serum sTREM-1 might be an important marker of sepsis, as well as bacterial and fungal pneumonia.[5,10] TREM-1 is also an amplifier of the inflammatory reaction. This probably explains the increase in TREM-1 or sTREM-1 expression found in non-infectious inflammatory disorders such as COPD, asthma, inflammatory bowel disease and acute pancreatitis.[11–14]

The role of sTREM-1 in airway inflammatory diseases, asthma and COPD, seems interesting not only in terms of the mechanisms of the inflammatory reaction. If TREM-1 is involved in airway inflammation, it might be hypothesized that it could be a marker of asthma or COPD. To date, there are no established and reliable biochemical factors that are used in the differential diagnosis or monitoring of asthma and COPD.[15] Two cytokines, IL-6 and IL-13, are probably the most effective in distinguishing these diseases. Sputum IL-6 level is increased in asthma and COPD compared to healthy subjects.[16] IL-6 has been shown to play a particular role in COPD: Elevated concentrations of it correlate with impaired lung function and are related to high frequencies of COPD exacerbations.[17] On the other hand, IL-13 is described as one of the key mediators of allergic asthma, promoting eosinophil predominance, goblet cell hyperplasia and mucus hypersecretion in the respiratory tract.[18] Elevated levels of IL-13 have been found in samples from the respiratory tract of asthma patients compared to healthy people. It has previously been shown that the ratio of IL-6 and IL-13 concentration in induced sputum is significantly different in asthmatics than in COPD patients.[19]

To date, the concentration of sTREM-1 in asthma and COPD patients has been evaluated in serum and plasma only. Systemic sTREM-1 levels correlated positively with asthma and COPD severity and negatively with lung function, and are a good marker of neutrophilic inflammation.[11,12,20] To the authors' knowledge, there have been no studies on sTREM-1 concentrations in respiratory samples from asthma and COPD patients. Also, the level of sTREM-1 has not been compared with other asthma or COPD biomarkers. The aims of this study were therefore (1) to evaluate sTREM-1 concentrations in BALF from asthma and COPD patients; and (2) to compare the utility of BALF sTREM-1 measurements in asthma/COPD differentiation with that of IL-6 and IL-13.

Material and methods

Characteristics of the patients

Eight patients with mild to moderate asthma (classified according to the Global Initiative for Asthma recommendations) and 11 patients with stage I-II COPD (classified according to the Global Initiative for Chronic Obstructive Lung Disease recommendations) were enrolled in the study.[21,22] All the patients were stable and had been free from exacerbations for at least 8 weeks before the start of the study. None of the patients had had respiratory infections for at least 1 month preceding the enrollment. The only concomitant respiratory inflammations in the participants were related to the upper airways: 6 patients (2 with asthma, 4 with COPD) had chronic sinusitis, and 4 patients (1 with asthma, 3 with COPD) had chronic rhinitis.

An important inclusion criterion was treatment with bronchodilators only, with no systemic or local anti-inflammatory treatment with corticosteroids. The only treatment used by the asthma patients before inclusion in the study were short-acting β2-agonists (SABAs) as needed (7 patients), and long-acting β2-agonists (LABAs) and SABAs as needed (one patient). In the COPD group, 6 patients were treated with SABAs only, 2 with long-acting muscarinic antagonists (LAMAs), 2 with LABAs and 1 with LAMAs and LABAs.

The study was approved by the Ethical Committee of the Medical University of Warsaw, and written informed consent was obtained from all the patients enrolled.

Bronchoalveolar lavage

Flexible bronchoscopies (using an Olympus BF-1T160 bronchoscope, Olympus Corporation, Tokyo, Japan) were performed under local anesthesia (2% lidocaine) after premedication with inhaled salbutamol (400 μg), intramuscular atropine (0.5 mg) and oral midazolam (7.5 mg). Supplemental oxygen was applied during the

entire procedure and blood oxygen saturation was monitored with a pulse oximeter (Pulsox DP-8, Konica Minolta Inc., Tokyo, Japan). Following macroscopic inspection of the bronchial tree, BAL was performed by wedging the bronchoscope in the bronchi of the middle lobe or the lingula and administering 4 × 50 mL sterile NaCl warmed to 37°C. Gentle suction was applied to recover the BALF into sterile containers. The subsequent steps of BALF processing were completed according to American Thoracic Society (ATS) recommendations.[23] The cells were evaluated and counted in May-Grünwald-Giemsa stained smears. The supernatants were stored at –70°C.

sTREM-1, IL-6 and IL-13 analysis

Soluble TREM-1, IL-6 and IL-13 concentrations in BALF were measured using appropriate ELISA kits (IQ Products, Groningen, Netherlands for sTREM-1; R&D Systems, Minneapolis, USA for IL-6 and IL-13) according to the manufacturers' instructions. The lower limits of detection were 7 pg/mL for sTREM-1, 0.76 pg/mL for IL-6 and 13.2 pg/mL for IL-13.

Statistical analysis

The results are presented as median and range of values. The statistical analysis was performed using STATISTICA 12.0 software (StatSoft Inc., Tulsa, USA). Differences between continuous variables were tested using Fisher's exact test or the nonparametric Mann-Whitney U test. Correlations between variables were analyzed with Spearman's rank test. Differences were considered statistically significant at $p < 0.05$.

Results

Clinical characteristics of the asthma and COPD patients

The basic comparative characteristics of the asthma and COPD patients are shown in Table 1. The patients with COPD were significantly older than the asthmatics. Spirometry results showed a significantly stronger impairment of respiratory function in COPD than in asthma.

Cellular composition of BALF in asthma and COPD

A significantly lower proportion of lymphocytes was found in the BALF of the COPD patients when compared to the asthmatics (Table 2).

Table 1. Demographic and clinical data of patients with asthma and COPD. The values are expressed as median and range of values; p-values were obtained using Fisher's exact test or the Mann-Whitney U test

Characteristics	Asthma	COPD	p-value
Gender (male/female)	6/2	8/3	0.664
Atopy (n)	7	5	0.079
Age (yrs)	43.5 (21–65)	65 (52–78)	0.0005
Smoking exposure (pack/years)	0 (0–21)	45 (30–100)	0.00002
Disease duration (months)	44.5 (12–264)	48.5 (12–216)	0.6574
FEV_1(% predicted)	92 (52–121)	73 (65–95)	0.0008
FVC (% predicted)	113 (87–143)	104 (83–123)	0.0327
FEV_1/FVC (%)	67 (49–90)	56 (43–64)	0.00005

COPD – chronic obstructive pulmonary disease, FEV_1 – forced expiratory volume at first second, FVC – forced vital capacity.

Table 2. The cellular composition of the bronchoalveolar lavage fluid (BALF) of asthma and COPD patients. The sum of all non-epithelial cells was counted as 100%. The values are expressed as median and range of values; p-values were obtained using the Mann-Whitney U test

BALF cells	Asthma	COPD	p-value
Macrophages (%)	84.5 (73–92)	92 (51–98)	0.146
Lymphocytes (%)	12 (7–16)	4 (1–25)	0.027
Neutrophils (%)	1.5 (1–7)	3.5 (1–23)	0.101
Eosinophils (%)	1.5 (0–11)	0 (0–4)	0.274

Comparison of BALF sTREM-1, IL-6 and IL-13 concentration in asthma and COPD

There were no significant differences in the BALF sTREM-1 concentration between asthma and COPD (Fig. 1). The median sTREM-1 concentration was 52.5 pg/mL (38.3–102.5 pg/ML) for asthma and 73.4 pg/mL (20.4––226.9 pg/mL) for COPD. No correlations between sTREM-1 concentration and the proportions of inflammatory cells in asthma or COPD were noted.

The median concentrations of IL-6 in BALF were: 14.3 pg/mL (0–62.6 pg/mL) and 24.6 pg/mL (7.8––75.5 pg/mL) for asthma and COPD respectively; and the median concentrations of IL-13 were 40.4 pg/mL (35.5–71.5 pg/mL) and 42.8 pg/mL (32.2–53.5 pg/mL) for asthma and COPD, respectively. The IL-6/IL-13 ratio was calculated for both groups. In 2 cases (from the COPD group) an undetectable level of IL-6 concentration was arbitrarily set as 0.76 pg/mL (the lower detection limit of the ELISA kit). IL-6/IL-13 ratio was significantly higher in the COPD (0.68 [0.15–1.55]) than in the asthma group (0.22 [0.02–0.99]) ($p = 0.043$) (Fig. 2).

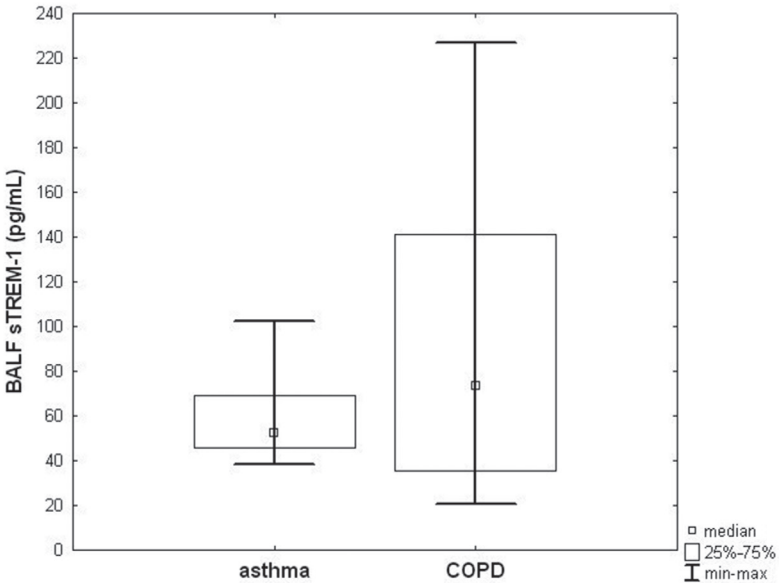

Fig. 1. sTREM-1 levels in the BALF of asthma and COPD patients. The values are expressed as median and range of values. The statistical analysis was performed using the Mann-Whitney U test

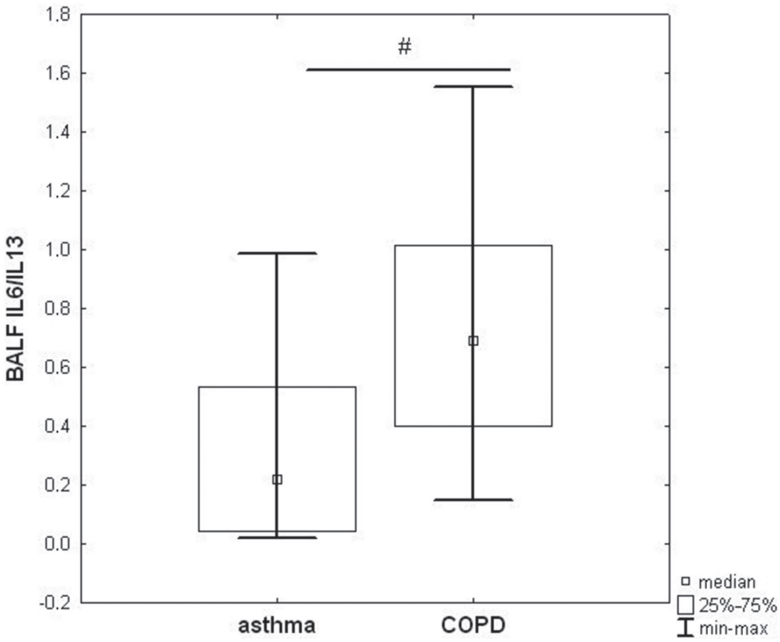

Fig. 2. The IL-6/IL-13 ratio in the BALF of asthma and COPD patients. The values are expressed as median and range of values. The statistical analysis was performed using the Mann-Whitney U test ($p < 0.05$)

Discussion

The study showed a measurable concentration of sTREM-1 in lavage fluid from peripheral airways. The results demonstrated that the level of sTREM-1 in BALF was not significantly different in asthma and COPD patients. This finding suggests that sTREM-1 is not suitable for the differentiation of obstructive lung diseases in their stable phase. Simultaneously, the study confirmed that measurement of other BALF biomarkers, IL-6 and IL-13, can more efficiently distinguish between asthma and COPD.

To the authors' knowledge, this is the first study comparing the BALF concentration of sTREM-1 in asthma and COPD patients. To date, only serum/plasma levels of sTREM-1 in asthma and COPD have been evaluated. Previous studies have demonstrated increased systemic sTREM-1 concentrations in asthma and COPD patients, and a relationship between serum sTREM-1 levels and the severity of the disease.[12,20] Bucova et al. found that in asthma patients, plasma sTREM-1 correlated with the clinical stage and disease severity, and was highly elevated during disease exacerbations.[12] On the other hand, a study by Rohde et al. that included COPD patients showed no differences in serum sTREM-1 levels between patients with advanced stable disease and those with COPD exacerbations.[11] As sTREM-1 is regarded as a marker of infection and a significant proportion of COPD exacerbations are related to airway infections, this finding seems surprising. The authors of the study concluded that the lack of any

increase in sTREM-1 during acute exacerbations may be explained by the high baseline levels of sTREM-1 caused by bacterial colonization present in patients with moderate to severe COPD, making any further increase during acute exacerbation COPD undetectable.

Serum levels of sTREM-1 in patients with asthma and COPD exacerbations were compared by Phua et al. Although the authors found no differences between the entire COPD and asthma groups, they noted a higher sTREM-1 level in Anthonisen type 1 COPD exacerbations compared to asthma and Anthonisen type 2 and 3 COPD exacerbations.[24] This seems to be consistent with the role of sTREM-1 in bacterial infections.

The results of the studies cited above prompted the present authors to investigate whether sTREM-1 concentrations in local respiratory samples (BALF) differ in patients with stable asthma and stable COPD. Interestingly, the BALF levels of sTREM-1 in the present study are comparable with serum/plasma sTREM-1 levels reported by other authors in mild stable asthma and COPD.[11,12] This may suggest that the systemic sTREM-1 level reflects the local expression of sTREM-1 in the respiratory tract. Although higher sTREM-1 concentrations were observed in the BALF of the COPD patients than in the asthma patients, the difference was not statistically significant. The lack of statistical significance may be at least partially related to the small number of patients in this study. Nevertheless, based on the preliminary data, it might be concluded that sTREM-1 level in lower airway respiratory samples does not discriminate stable asthma and COPD. Since, to the authors' knowledge, the BALF sTREM-1 levels in stable asthma and COPD have not been reported before, no comparison of the current results with other studies is possible.

In light of the existing data, the major clinical application of sTREM-1 measurement seems to be the differentiation between bacterial and viral infections.[11,25] This might be of particular interest in airway and pulmonary infections. Shu et al. reported that sTREM-1 may differentiate between mycobacterial colonization and active mycobacterial pulmonary disease.[26] Because of potential relationships between airway infections and chronic obstructive airway diseases, the measurement of sTREM-1 concentrations in respiratory samples seems to have both clinical and research applications. It has been shown that the microbial composition of the airways may be an important determinant of the risk of developing asthma or COPD.[27] If sTREM-1 is a sensitive marker of microbial colonization in the respiratory tract, this may add some new data on the pathogenesis of obstructive airway diseases. The need for a sensitive biomarker of bacterial infection might be exemplified by the fact that over 70% of the bacterial species on body surfaces cannot be cultured by currently available techniques.[28] The results of the present study suggest that the microbial burden in stable mild-to-moderate asthma and COPD is similar. The relationship between the sTREM-1 concentration in the lungs and microbiological dependency in asthma and COPD certainly needs further investigation.

To compare BALF sTREM-1 levels with other recognized asthma and COPD biomarkers this study also investigated the BALF concentrations of 2 cytokines – IL-6 and IL-13. It has previously been reported that concentrations of IL-6 are increased in COPD, whereas IL-13 is elevated in asthma.[29,30] However, IL-6 and IL-13 are not specific for a single disease. Therefore, it was assumed that the IL-6/IL-13 ratio characterizes asthma and COPD more accurately than the levels of either single cytokine, since the level of an individual cytokine may change dynamically in the course of the disease.[19] The results obtained supported this hypothesis. The IL-6/IL-13 ratio in BALF was significantly different in asthma than in COPD, even though there was no difference between BALF sTREM-1 levels in these 2 diseases.

The study has some limitations. The major flaw is the small number of patients included in the study. This was due to the preliminary nature of the research and to the relatively invasive bronchoscopic procedure required to collect BALF. The small number of patients did not permit a further sub-analysis of sTREM-1 levels in patients with different asthma and COPD phenotypes. The fact that only one type of biological sample was used in the study might be considered a second limitation. It could have been interesting to compare BALF sTREM-1 concentrations with sTREM-1 levels in induced sputum, exhaled breath condensate and serum. Third, since the study did not include a control group, no comparison could be made between BALF sTREM-1 concentrations in patients with obstructive pulmonary diseases and in healthy subjects.

Conclusions

The study showed that BALF sTREM-1 levels are similar in mild-to-moderate asthma and COPD. In contrast, the IL-6/IL-13 ratio measured in BALF effectively differentiated asthma and COPD. Further studies that involve patients with different asthma and COPD phenotypes and different degrees of disease severity are warranted to elucidate whether BALF sTREM-1 concentrations might be considered one of the local inflammatory markers in obstructive lung diseases.

References

1. Klesney-Tait J, Turnbull IR, Colonna M. The TREM receptor family and signal integration. *Nat Immunol*. 2006;7:1266–1273.
2. Read CB, Kuijper JL, Hjorth SA, et al. Cutting edge: Identification of neutrophil PGLYRP1 as a ligand for TREM-1. *J Immunol*. 2015;194:1417–1421.
3. Bleharski JR, Kiessler V, Buonsanti C, et al. A role for triggering receptor expressed on myeloid cells-1 in host defense during the early-induced and adaptive phases of the immune response. *J Immunol*. 2003;170:3812–3818.

4. Radsak MP, Salih HR, Rammensee HG, Schild H. Triggering receptor expressed on myeloid cells-1 in neutrophil inflammatory responses: Differential regulation of activation and survival. *J Immunol*. 2004;172:4956–63.
5. Gibot S, Cravoisy A, Kolopp-Sarda MN, et al. Time-course of sTREM (soluble triggering receptor expressed on myeloid cells)-1, procalcitonin, and C-reactive protein plasma concentrations during sepsis. *Crit Care Med*. 2005;33:792–796.
6. Masekela R, Anderson R, de Boeck K, et al. Expression of soluble triggering receptor expressed on myeloid cells-1 in childhood CF and non-CF bronchiectasis. *Pediatr Pulmonol*. 2015;50:333–339.
7. Gómez-Piña V, Soares-Schanoski A, Rodríguez-Rojas A, et al. Metalloproteinases shed TREM-1 ectodomain from lipopolysaccharide-stimulated human monocytes. *J Immunol*. 2007;179:4065–4073.
8. Mahdy AM, Lowes DA, Galley HF, Bruce JE, Webster NR. Production of soluble triggering receptor expressed on myeloid cells by lipopolysaccharide-stimulated human neutrophils involves de novo protein synthesis. *Clin Vaccine Immunol*. 2006;13:492–495.
9. Lemarié J, Barraud D, Gibot S. Host response biomarkers in sepsis: Overview on sTREM-1 detection. *Methods Mol Biol*. 2015;1237:225–239.
10. Gibot S, Cravoisy A, Levy B, Bene MC, Faure G, Bollaert PE. Soluble triggering receptor expressed on myeloid cells and the diagnosis of pneumonia. *N Engl J Med*. 2004;350:451–458.
11. Rohde G, Radsak MP, Borg I, Buhl R, Schultze-Werninghaus G, Taube C. Levels of soluble triggering receptor expressed on myeloid cells 1 in infectious exacerbations of chronic obstructive pulmonary disease. *Respiration*. 2012;83:133–139.
12. Bucova M, Suchankova M, Dzurilla M, et al. Inflammatory marker sTREM-1 reflects the clinical stage and respiratory tract obstruction in allergic asthma bronchiale patients and correlates with number of neutrophils. *Mediators Inflamm*. 2012;2012:628754.
13. Park JJ, Cheon JH, Kim BY, et al. Correlation of serum-soluble triggering receptor expressed on myeloid cells-1 with clinical disease activity in inflammatory bowel disease. *Dig Dis Sci*. 2009;54:1525–1531.
14. Yasuda T, Takeyama Y, Ueda T, et al. Increased levels of soluble triggering receptor expressed on myeloid cells-1 in patients with acute pancreatitis. *Crit Care Med*. 2008;36:2048–2053.
15. Röpcke S, Holz O, Lauer G, et al. Repeatability of and relationship between potential COPD biomarkers in bronchoalveolar lavage, bronchial biopsies, serum, and induced sputum. *PloS One*. 2012;7:e46207.
16. Ravi AK, Khurana S, Lemon J, et al. Increased levels of soluble interleukin-6 receptor and CCL3 in COPD sputum. *Respir Res*. 2014;15:103.
17. Bhowmik A, Seemungal TA, Sapsford RJ, Wedzicha JA. Relation of sputum inflammatory markers to symptoms and lung function changes in COPD exacerbations. *Thorax*. 2000;55:114–120.
18. Wills-Karp M, Luyimbazi J, Xu X, et al. Interleukin-13: Central mediator of allergic asthma. *Science*. 1998;282:2258–2261.
19. Grubek-Jaworska H, Paplińska M, Hermanowicz-Salamon J, et al. IL-6 and IL-13 in induced sputum of COPD and asthma patients: correlation with respiratory tests. *Respiratio*n. 2012;84:101–7.
20. Radsak MP, Taube C, Haselmayer P, et al. Soluble triggering receptor expressed on myeloid cells 1 is released in patients with stable chronic obstructive pulmonary disease. *Clin Dev Immunol*. 2007; 2007:52040.
21. Global Initiative for Asthma. Global strategy for asthma management and prevention. www.ginaasthma.com. 2010 update. Accessed February 12 2016.
22. Global Initiative for Obstructive Lung Disease. Global strategy for the diagnosis, management and prevention of chronic obstructive pulmonary disease. www.goldcopd.org. 2010 update. Accessed February 12 2016.
23. Meyer KC, Raghu G, Baughman RP. An official American Thoracic Society clinical practice guideline: The clinical utility of bronchoalveolar lavage cellular analysis in interstitial lung disease. *Am J Respir Crit Care Med*. 2012;185:1004–1014.
24. Phua J, Koay ESC, Zhang D, et al. Soluble triggering receptor expressed on myeloid cells-1 in acute respiratory infections. *Eur Respir J*. 2006;28:695–702.
25. ten Oever J, Tromp M, Bleeker-Rovers CP, et al. Combination of biomarkers for the discrimination between bacterial and viral lower respiratory tract infections. *J Infect*. 2012;65:490–495.
26. Shu CC, Lee LN, Wu MF, et al. Use of soluble triggering receptor expressed on myeloid cells-1 in non-tuberculous mycobacterial lung disease. *Int J Tuberc Lung Dis*. 2011;15:1415–1420.
27. Han MK, Huang YJ, Lipuma JJ, et al. Significance of the microbiome in obstructive lung disease. *Thorax*. 2012;67:456–463.
28. Suau A, Bonnet R, Sutren M, et al. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Appl Environ Microbiol*. 1999;65: 4799–4807.
29. Donaldson GC, Seemungal TAR, Patel IS, et al. Airway and systemic inflammation and decline in lung function in patients with COPD. *Chest*. 2005;128:1995–2004.
30. Komai-Koma M, McKay A, Thomson L, et al. Immuno-regulatory cytokines in asthma: IL-15 and IL-13 in induced sputum. *Clin Exp Allergy*. 2001;31:1441–1448.

The coexistence of autoimmune rheumatic diseases and thymomas

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Abstract

Background. Autoimmune rheumatic diseases (ARDs), involving immune disturbances resulting from auto-inflammatory mechanisms, are a group of diseases characterized by autoimmunity and autoimmune-mediated organ damage. Thymoma, whose mechanism is also associated with immune abnormalities, is the most common neoplasm of the anterior mediastinum. But thymoma with ARDs is relatively less frequent. The clinical characteristics of the coexistence of ARDs and thymomas are still not very clear. And the therapeutic strategy for ARDs combined with thymomas varies, with an uncertain outcome.

Objectives. The aim of this study was to investigate the clinical characteristics of the coexistence of ARDs and thymomas in order to speculate whether a thymectomy is effective for ARDs combined with thymomas, and to seek the proper therapeutic strategy for treating ARDs combined with thymomas.

Material and methods. We presented 2 cases of the coexistence of ARDs and thymomas. Then, we summarized 20 cases (including our 2 cases) in which the ARD was diagnosed concurrently with, or prior to, the thymoma.

Results. Pure red cell aplastic anemia (PRCA) might be associated with an ARD and a thymoma, and a thymectomy may lead to the appearance, exacerbation, or remission of ARDs.

Conclusions. Searching for a thymoma is necessitated if a patient with ARDs experiences PRCA and the effects of thymectomy in ARDs combined with thymomas may be associated with the onset sequence of ARDs and thymomas.

Key words: autoimmune rheumatic diseases, thymoma, PRCA, thymectomy

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Autoimmune rheumatic diseases (ARDs) are a group of rare, heterogeneous disorders characterized by autoimmunity and autoimmune-mediated organ damage. They affect an estimated 7.6–9.4% of the population worldwide, and they include systemic lupus erythematosus (SLE), Sjögren's syndrome (pSS), rheumatoid arthritis (RA), dermatomyositis (DM), and systemic sclerosis (SSc), etc.[1] Thymoma is the most common tumor in the anterior mediastinum.[2] However, the combination of an ARD and a thymoma in the same patient is relatively less frequent. Herein, we will present 2 ARD cases that also simultaneously had a thymoma.

Case 1

A 60-year-old Chinese woman was diagnosed with SLE in 2005 at another hospital because she had polyarthritis, alopecia, neutropenia, and low complete C3/C4 levels, along with positive antinuclear antibody and anti-dsDNA antibody tests. She was 1st treated with prednisone (50 mg/day), wilfordii (60 mg/day), and methotrexate (10 mg/week), and her maintenance treatment included prednisone (10 mg/day) for a long time while her disease was stable. She was admitted to our hospital in March 2014 because of dizziness, fatigue, and mild shortness of breath that had persisted for 3 months. A physical examination revealed a pale appearance and mild lower extremity edema. Laboratory findings were as follows: hemoglobin, 30 g/L; ANA, positive, (with a titer of 1 : 160; granular pattern); anti-dsDNA antibody, negative; anti-histone antibody and antiribosomal protein antibodies, positive; complement C3 and C4, 483.0 mg/L and 55.3 mg/L, respectively; and Coombs and Ham tests, negative. The albumin level was 30.9 g/L. A bone marrow cytology test performed in another hospital was as follows: active bone marrow hyperplasia; granulocyte prolifera-

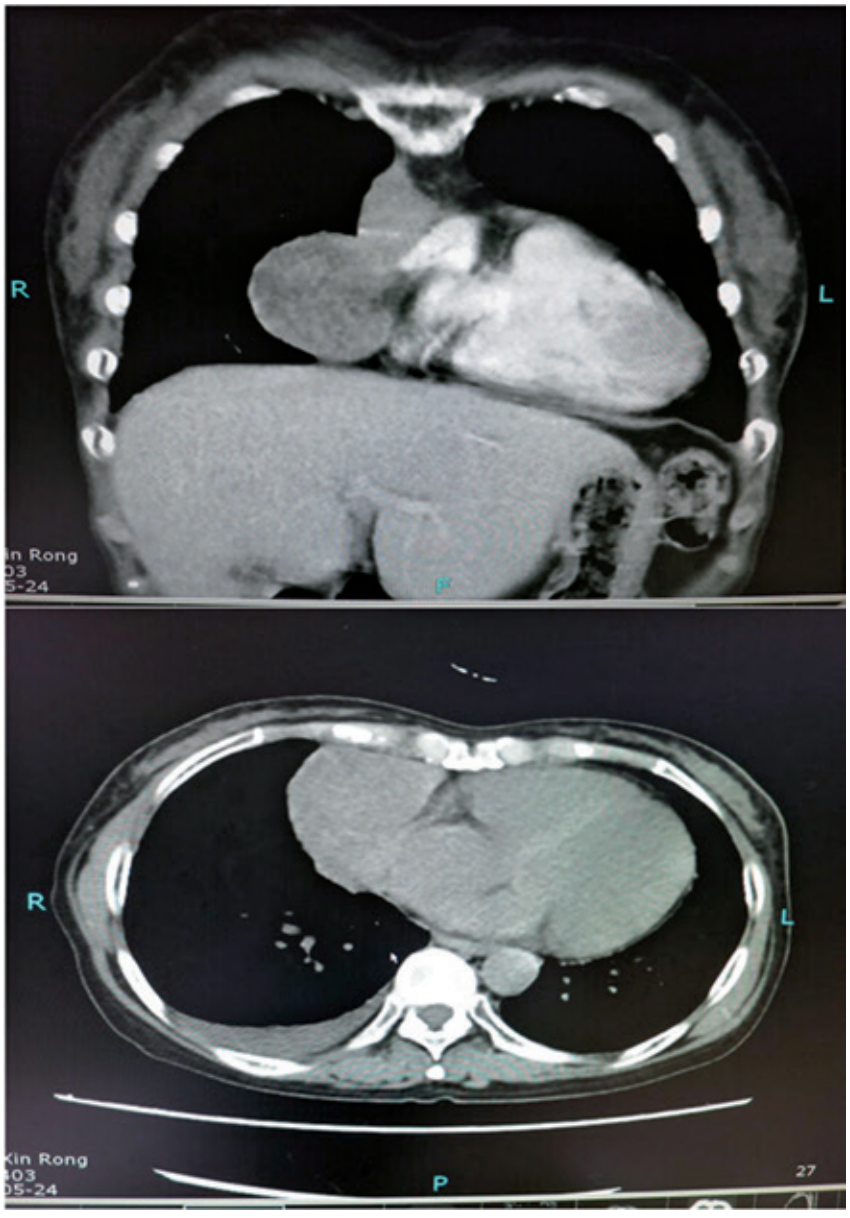

Fig. 1. Computed tomography of the chest showing a mass in the anterior mediastinum (case 1)

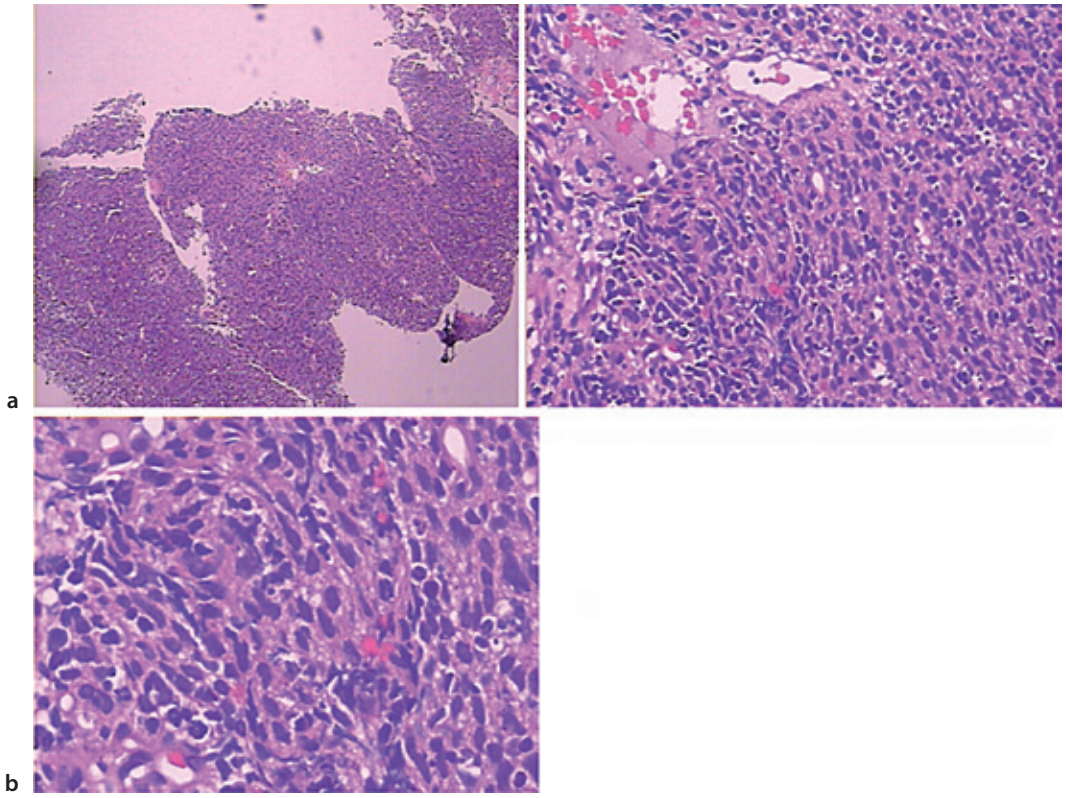

Fig. 2. a) Low-power photomicrograph of the thymic mass (H & E, ×40); b) middle-power photomicrograph of the thymic mass (H & E, ×200); c) higher-power photomicrograph of the thymic mass (H & E, ×400) (case 1)

tion; and significantly reduced red blood cell hyperplasia. The megakaryocytes were normal and the platelets were distributed in clusters. The peripheral blood slides were normal. Pure red cell aplastic anemia (PRCA) was diagnosed. A chest computed tomography (CT) scan revealed a mass in the right anterior mediastinum that measured 68 mm × 62 mm (Fig. 1), with a right pleural effusion. A CT-guided percutaneous needle biopsy of the mass was performed on March 28. The pathological result showed a type B3 thymoma (Fig. 2), according to the World Health Organization (WHO) classification. A thymectomy was not performed because she had severe anemia. She was treated as follows: methylprednisolone (40 mg/day), hydroxychloroquine (0.2 g/day), and regular blood transfusions. Hydroxychloroquine (0.2 g/day) and regular blood transfusions were continued, and for daily maintenance, methylprednisolone was gradually reduced to 12 mg. Upon her follow-up, her disease was deemed to be stable.

Case 2

A 63-year-old Chinese woman with a history of hypothyroidism was diagnosed with Sjögren's syndrome in 2007 at our hospital based on dry mouth, dry eyes, positive antinuclear antibody and anti-SSA/SSB tests, and positive corneal fluorescein staining, along with a tear film break-up time of 3 s. Anti-Sm antibody and anti-dsDNA antibody tests were both negative. A urinalysis was negative for proteinuria, and a chest radiograph was normal at that time. She was treated with methylprednisolone (40 mg/day), wilfordii (60 mg/day), leflunomide (10 mg/day), and levothyroxine (100 μg/day) for her illness. This treatment protocol was continued, and for daily maintenance, methylprednisolone was gradually reduced to 4 mg. The disease was stable during follow-up. The patient was admitted to our hospital again because of lower limb edema on August 19, 2014.

Upon admission, a physical examination revealed 4 dental caries with a residual root only and lower extremity edema. The laboratory findings were as follows: blood cell counts, normal; proteinuria, 3+; 24-h urinary protein, 3.43 g; globulin, 51.60 g/L; albumin, 27.50 g/L; triglyceride, 1.58 mg/L; cholesterol, 6.27 mmoL/L; ANA titer, 1 : 320 (homogeneous pattern); anti-SSA and anti-SSB tests, positive; and anti-Sm antibody and anti-dsDNA antibody tests, negative. A chest CT revealed a large mass measuring 39 mm × 31 mm in the area of the thymus (Fig. 3).

A thymectomy was performed on August 26, 2014. A type B3 thymoma was considered according to the WHO classification, and the tumor did not involve any other structures (Fig. 4). More than 1 month after the thymectomy, the patient was admitted to our hospital again because of exacerbation of lower extremity edema; the laboratory values were as follows: Blood cell counts, normal; proteinuria, 3+; globulin, 28.60 g/L; albumin, 24.08 g/L; triglycerides, 1.58 mg/L; cholesterol, 7.70 mmoL/L; ANA titer, 1 : 320 (homogeneous pattern);

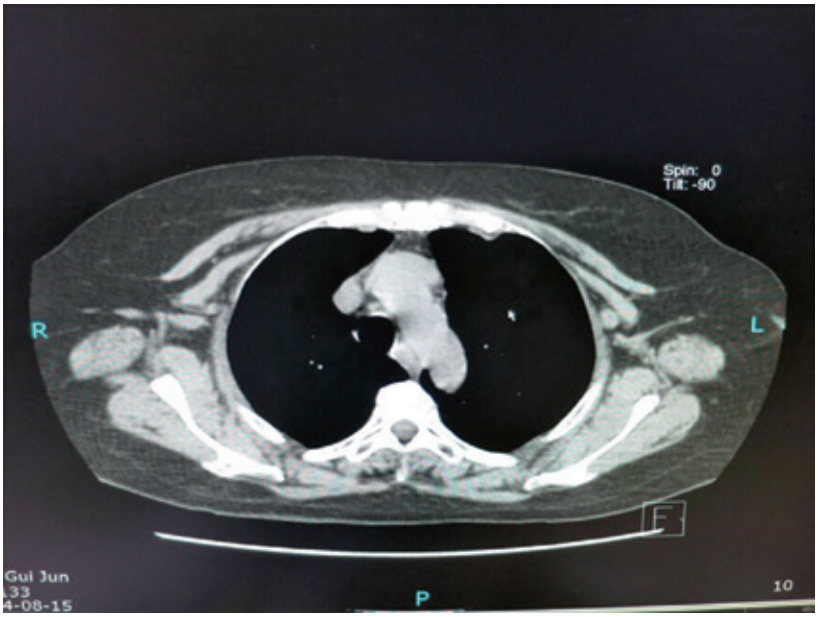

Fig. 3. Computed tomography of the chest showing a mass in the right anterior mediastinum (case 2)

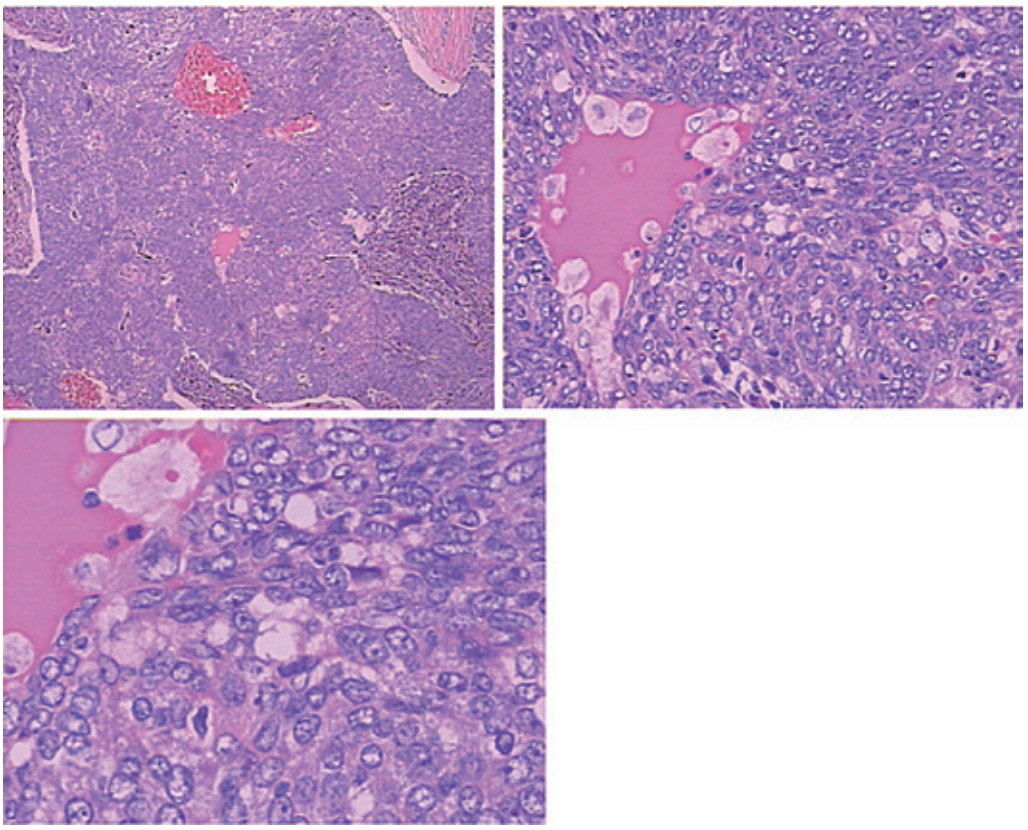

Fig. 4. a) Low-power photomicrograph of the thymic mass (H & E, ×40); b) middle-power photomicrograph of the thymic mass (H & E, ×200); c) higher-power photomicrograph of the thymic mass (H & E, ×400) (case 2)

and anti-SSA and anti-SSB tests, still positive. A color Doppler ultrasound revealed thromboses in the external iliac vein, right femoral vein, and popliteal vein. Then, anticoagulant and thrombolytic therapy was administered (low-molecular-weight heparin calcium [4100u BID ip], urokinase [20wu QD IV gtt], and argatroban [20 mg BID IV gtt]). The lower extremity edema gradually subsided. After that, the patient was treated with rivaroxaban (10 mg QD po) as a maintenance anticoagulant treatment.

Discussion

ARDs are a group of diseases involving immune disturbances resulting from auto-inflammatory mechanisms that are frequently the underlying cause.[1] A thymoma is the most frequent tumor of the thymus, the mechanism of which is also associated with immune abnormalities.[2] Structural and functional changes in the thymus may lead to the loss of self-tolerance and may occur with autoim-

mune diseases.[2] There are 3 morphological types of thymomas based on genetic alterations according to 2004 WHO classifications: A, B (B1, B2, and B3), and AB. Carcinoma of the thymus is designated as type C. Compared with type A, B1, B2, and AB, the B3 subtype has high 15-year recurrence rates and a poor prognosis.[3] A thymoma is commonly associated with a variety of systemic and autoimmune disorders such as PRCA, hypogammaglobulinemia, pancytopenia, collagen diseases, and most commonly, myasthenia gravis (MG); sometimes 2 or even 3 autoimmune diseases coexist.[4]

In recent years, there have been a few case reports regarding the coexistence of ARDs and thymomas, such as SLE, RA, DM, SSc, and pSS.[5–20] Several reports documented the appearance of ARD after a thymectomy for a thymoma. Other reports described the appearance of a thymoma years after a patient was diagnosed with an ARD, as in our 2 cases. In some cases, the 2 diagnoses were concurrent. Herein, we have summarized 20 cases (including our 2 cases) and have included relatively complete information. For all 20 cases, the ARD was diagnosed concurrently with or prior to the thymoma (Table 1).

The association of a thymoma with an ARD is not frequent. In clinical studies, the prevalence of SLE in patients with a thymoma varied between 1.5 and 2%.[16] PRCA has been associated with a thymoma or ARD. Approximately 2–5% of patients with a thymoma will have PRCA.[21] PRCA could also arise as a complication of an ARD such as SLE, but it is very rare.[22,23] However, the coexistence of all 3 disorders – an ARD, a thymoma, and PRCA – in the same patient is extremely rare. To our knowledge, there have been 6 previous reports of cases with an ARD, a thymoma, and PRCA.[12,19,24–27] However, for only 2 of them, an ARD was diagnosed concurrently or prior to a thymoma and PRCA.[12,19] Herein, we reported on a case (case 1) of a patient with the SLE–thymoma–PRCA triad. The patient developed PRCA and a thymoma 9 years after being diagnosed with SLE. All the patients in the 3 cases were females aged 48, 60, and 63 years, and they had SLE for 3, 9, and 9 years, respectively. Therefore, PRCA might be associated with an ARD and a thymoma, particularly in older patients who have an ARD disease course with a long duration. We speculate that PRCA may be associated with a thymoma, although there is no significant relationship between the occurrence of PRCA and the pathological type of thymoma. Searching for a thymoma is necessitated if a patient with ARD experiences PRCA.

The therapeutic strategy for ARDs combined with thymomas varies, with an uncertain outcome. Underlying conditions involving ARDs and thymomas should be treated. A thymectomy or gamma irradiation of the thymus gland may be performed for a thymoma. Neither chemotherapy nor irradiation is beneficial for the treatment of ARDs.[8,28] The immunologic effects of a thymectomy for an ARD are also not clear. Several reports have documented the appearance, exacerbation, or remission of an ARD after a thymectomy.[15] Of all the cases reviewed in this report, almost all 9 patients who were diagnosed ARD and a thymoma at the same time achieved remission through either surgery or radiation.[8–11,13–16,18] In one of these cases, the patient with pSS was deemed to be in remission with surgery alone.[18] However, for the patients who were diagnosed with an ARD before the thymoma, none achieved remission with surgery alone. Medicines such as corticosteroids, cyclophosphamide, or cyclosporine should also be used to treat ARDs. In spite of this, most patients experience an exacerbation, or their disease becomes refractory to treatment.[6,7,11,12] As for our 1st case, the patient did not undergo surgery, and her SLE and PRCA were deemed stable. As for our 2nd case, the patient's condition worsened secondary to massive proteinuria and vein thrombosis several months after the thymectomy. It has also been reported that patients with SLE, a thymoma, and PRCA died of a pulmonary embolism after having a thymectomy.[27] Therefore, a thymectomy or radiation would be effective for helping patients achieve remission with an ARD if the ARD were concurrently diagnosed with a thymoma; however, if the ARD were diagnosed prior to the thymoma, then in addition to treatment for the thymoma, a corticosteroid or immunological inhibitor should be used to help obtain remission for the ARD. Sometimes rituximab, donor lymphocyte infusion (DLI), or hematopoietic stem cell transplantation (HSCT) could also be used to treat the complication, PRCA.[19,29] We surmise that if a thymoma and an ARD occur simultaneously, the thymoma might exert a role in the development of ARDs, as seen with MG. A thymectomy or radiation might be helpful for achieving remission with an ARD. If the thymoma were to occur several years after the ARD, it would not be clear if the thymoma was the primary abnormality or the immunologic abnormalities seen in patients with an ARD would lead to thymus disorders and tumor development. A thymectomy or radiation is not always associated with remission from an ARD and might sometimes cause an exacerbation. It is necessary to study additional cases to elucidate the effects of thymectomy in ARDs combined with thymomas and to help provide additional guidance for treatment.

In conclusion, we reported 2 cases and compiled a series of cases involving an ARD combined with a thymoma. The data indicated that ARDs can coexist with thymomas and that a thymoma should be included in the differential diagnosis if a patient with an ARD complains of PRCA. The result of a thymectomy for a patient with an ARD can be unpredictable. Indeed, ARDs involve a multisystem disease with heterogeneous and varied manifestations. Therefore, study of additional cases is necessitated for exploring issues related to ARDs and thymomas.

Table 1. ARDs with thymomas. Review of literature

Report	Age at ARD diagnosis (y)/sex	Age at diagnosis of thymoma (y)	ARD	PRCA	Thymoma pathological type	Treatment of thymoma	Treatment of ARD	Outcome of ARD
Simeone JF (1975)[5]	not reported/f	1 year later	SLE	no	invasive lymphoepithelial	surgery + radiation	no treatment	remission
Steven MM (1984)[6]	48/f	50	SLE	no	not reported	surgery + radiation	methylprednisolone	exacerbation
Steven MM (1984)[6]	48/f	49	SLE	no	invasive	radiation + surgery	methylprednisolone.	exacerbation
Ben-Shahar M (1987)[7]	65/m	> 65	PSSC	no	malignant	surgery	chronic hemodialysis	exacerbation
Parakova Z (1992)[8]	62/f	62	SLE	no	benign	surgery	prednisolone	stable
Menon S (1993)[9]	62/f	62	SLE	no	not reported	surgery	hydroxychloroquine	remission
Sakuragi T (1995)[10]	59/f	59	PSSC	no	epithelial type	surgery	steroid	remission
Zandman-Goddard G (1995)[11]	45/f	48	SLE	no	benign	surgery	steroid	refractory
Zandman-Goddard G (1995)[11]	30/f	30	SLE	no	not reported	surgery	not reported	remission
Duchmann R (1995)[12]	54/f	63	SLE	yes	not reported	surgery	not reported	exacerbation
Matsumoto Y (1996)[13]	19/f	19	pSS	no	benign (lymphoepithelial)	surgery + radiation	prednisolone	remission
Ago T (1999)[14]	22/f	22	DM	no	invasive	surgery	methylprednisolone	remission
Bozzolo E (2000)[15]	27/f	27	SLE	no	mixed lymphocytic epithelial	surgery	prednisone and hydroxychloroquine	remission
Boonen A (2000)[16]	76/f	76	SLE	no	non-invasive spindle cell	surgery	no treatment	remission and then exacerbation 4 weeks later
Lin YC (2006)[17]	59/f	60	MCTD	no	IVb (Masaoka staging system)	radiation	prednisolone and hydroxychloroquine	remission
Tsai Y (2013)[18]	63/m	63	pSS	no	type A	surgery	no treatment	remission
Marmont AM (2014)[19]	45/f	48	SLE	yes	nodular epithelial	radiation	fludarabine and CYC, DLI and allo- HSCT	remission
Zou L (2014)[20]	27/f	38	RA	no	not reported	not reported	prednisone, amethopterin, sulfasalazine, hydroxychloroquine	remission
Present case 1	51/f	60	SLE	yes	type B3	no treatment	methylprednisolone, hydroxychloroquine and regular blood transfusions	stable
Present case 2	56/f	63	pSS	no	type B3	surgery	methylprednisolone, wilfordine, anticoagulant	exacerbation

RA – rheumatoid arthritis; SLE – systemic lupus erythematosus; pSS – Sjögren's syndrome; MCTD – mixed connective tissue disease; DM – dermatomyositis; PSSC – progressive systemic sclerosis; DLI – donor lymphocyte infusion; CYC – cyclophosphamide; HSCT – hematopoietic stem cell transplantation.

References

1. Goldblatt F, O'Neill SG. Clinical aspects of autoimmune rheumatic diseases. *Lancet*. 2013;382(9894):797–808.
2. Shelly S, Agmon-Levin N, Altman A, Shoenfeld Y. Thymoma and autoimmunity. *Cell Mol Immunol*. 2011;8(3):199–202.
3. Strobel P, et al. Thymoma and thymic carcinoma: An update of the WHO Classification 2004. *Surg Today*. 2005;35(10):805–811.
4. Mejri N, Chabchoub I, Gargouri I. Effect of chemotherapy on autoimmune hepatitis in thymoma: A case report and literature review. *Cancer Biol Med*. 2013;10(3):169–173.
5. Simeone JF, McCloud T, Putman CE, Marsh J. Thymoma and systemic lupus erythematosus. *Thorax*. 1975;30(6):697–700.
6. Steven MM, Westedt ML, Eulderink F, Hazevoet HM, Dijkman JH, Cats A. Systemic lupus erythematosus and invasive thymoma: Report of two cases. *Ann Rheum Dis*. 1984;43(6):825–828.
7. Ben-Shahar M, Rosenblatt E, Green J, Cohen I. Malignant thymoma associated with progressive systemic sclerosis. *Am J Med Sci*. 1987, 294(4), p. 262267.
8. Parakova Z, Hrncir Z, Nozicka Z. Thymoma in the anterior mediastinum and systemic lupus erythematosus. *Vnitr Lek*. 1992;38(2):177–183.
9. Menon S, Snaith ML, Isenberg DA. The association of malignancy with SLE: An analysis of 150 patients under long-term review. *Lupus*. 1993;2(3):177–181.
10. Sakuragi T, Furukawa T, Yonemura T, Yoshida T, Irie K. Thymoma associated with progressive systemic sclerosis: A case report. *Nihon Kyobu Geka Gakkai Zasshi*. 1995;43(1):135–138.
11. Zandman-Goddard G, Lorber M, Shoenfeld Y. Systemic lupus erythematosus and thymoma: A double-edged sword. *Int Arch Allergy Immunol*. 1995;108(1):99–102.
12. Duchmann R, Schwarting A, Poralla T, Meyer zum Büschenfelde KH, Hermann E. Thymoma and pure red cell aplasia in a patient with systemic lupus erythematosus. *Scand J Rheumatol*. 1995;24(4):251–254.
13. Matsumoto Y, Hirai S, Ohashi M, Imaizumi M. Sjogren's syndrome associated with thymoma. *J Am Acad Dermatol*. 1996;35(4):639–640.
14. Ago T, Nakamura M, Iwata I, et al. Dermatomyositis associated with invasive thymoma. *Intern Med*. 1999;38(2):155–159.
15. Bozzolo E, Bellone M, Quaroni N, Voci C, Sabbadini MG. Thymoma associated with systemic lupus erythematosus and immunologic abnormalities. *Lupus*. 2000;9(2):151–154.
16. Boonen A, Rennenberg R, van der Linden S. Thymoma-associated systemic lupus erythematosus, exacerbating after thymectomy. A case report and review of the literature. *Rheumatology (Oxford)*. 2000;39(9):1044–1046.
17. Lin YC, Wu CC, Ou TT, Yen JH, Liu HW, Tsai WC. Malignant thymoma associated with mixed connective tissue disease: A case report. *Clin Rheumatol*. 2006;25(2):262–264.
18. Tsai Y, Lin Y, Chen C, Tzao C. Thymoma associated with myasthenia gravis and Sjogren syndrome. *West Indian Med J*. 2013;62(3):264–265.
19. Marmont AM, Bacigalupo A, Gualandi F, Bregante S, van Lint MT, Geroldi S. Systemic lupus erythematosus complicated with thymoma and pure red cell aplasia (PCRA): CR of both complications following thymectomy and allogeneic haematopoietic SCT (HSCT), but persistence of antinuclear antibodies (ANA). *Bone Marrow Transplant*. 2014;49(7):982–983.
20. Zou L, Xiong Z, Dun Y, Xiong Y. A case of rheumatoid arthritis associated with Hashimoto's thyroiditis and thymoma. *Int J Rheum Dis*. 2014. doi: 10.1111/1756-185X.12453.
21. Chintakuntlawar AV, Rizvi SA, Cassivi SD, Pardanani A. Thymoma-associated pancytopenia: Immunosuppressive therapy is the cornerstone for durable hematological remission. *Ann Hematol*. 2015; 94(3):453–458.
22. Habib GS, Saliba WR, Froom P. Pure red cell aplasia and lupus. *Semin Arthritis Rheum*. 2002;31(4):279–283.
23. Koyama, RV, Silva LF, Henriques VB, Tran C, Yoshikawa GT. Pure red cell aplasia associated with systemic lupus erythematosus. *Acta Reumatol Port*. 2014;39(3):265–268.
24. Fallahi S, Akbarian M, Dabiri S. Pure red cell aplasia as a presenting feature in systemic lupus erythematosus and association with thymoma, hypothyroidism and hypoparathyroidism: A case report and literature review. *Iran J Allergy Asthma Immunol*. 2014;13(2): 138–143.
25. Mankia S, Giles IP. Pure red cell aplasia in a patient with lupus-like disease and thymoma. *Grand Rounds*. 2011;11(1):37–40.
26. MacKechnie HL, Squires AH, Platts M, Pruzanski W. Thymoma, myasthenia gravis, erythroblastopenic anemia and systemic lupus erythematosus in one patient. *Can Med Assoc J*. 1973;109(8):733–738.
27. Iwadate H, Kobayashi H, Shio K, et al. A case of systemic lupus erythematosus complicated by pure red cell aplasia and idiopathic portal hypertension after thymectomy. *Mod Rheumatol*. 2006;16(2): 109–112.
28. Rosman A, Atsumi T, Khamashta MA, Ames PR, Hughes GR. Development of systemic lupus erythematosus after chemotherapy and radiotherapy for malignant thymoma. *Br J Rheumatol*. 1995;34(12): 1175–1176.
29. Gupta RK, Ezeonyeji AN, Thomas AS, Scully MA, Ehrenstein MR, Isenberg DA. A case of pure red cell aplasia and immune thrombocytopenia complicating systemic lupus erythematosus: Response to rituximab and cyclophosphamide. *Lupus*. 2011;20(14):1547–1550.

Vascular endothelial growth factor (VEGF)-C, VEGF-D, VEGFR-3 and D2-40 expressions in primary breast cancer: Association with lymph node metastasis

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Abstract

Background. Two members of the vascular endothelial growth factor (VEGF) family, VEGF-C and -D, are known as lymphangiogenic growth factors and play an important role in tumor lymphangiogenesis via activation of the VEGF receptor (VEGFR)-3, which is expressed in lymphatic endothelial cells. D2-40 is a specific antibody for lymphatic vessel density (LVD).

Objectives. In the present study, we have aimed to evaluate whether intra- and peri-tumoral D2-40-positive lymphatic vessels affect lymph node metastasis and to investigate the relationship between LVD and lymph node metastasis in breast cancer.

Material and methods. We have evaluated the relationships between lymph node metastasis and VEGF-C, VEGF-D, VEGFR-3 and D2-40 expressions in breast cancer cells using immunohistochemistry. VEGF-C, VEGF-D and VEGFR-3 expression were found in tumor cells in the majority of the cases (83.75, 97.5 and 95%, respectively).

Results. There was a significant positive relationship between VEGF-D expression and lymph node metastasis ($p < 0.05$) however no significant association was found in VEGF-C and VEGFR-3 expressions. It was found that patients with high-expression of VEGF-D have a high level of both peri- and intra-tumoral LVD compared to those with low expression of VEGF-D ($p < 0.05$).

Conclusions. Our results support that examination of VEGF-D expression in breast cancer cells may be beneficial in the identification of lymph node metastasis.

Key words: breast cancer, metastasis, VEGF, D2-40

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Breast cancer is the most commonly diagnosed cancer in Turkish women as well as women worldwide.[1] According to GLOBOCAN 2012, the most common cause of cancer mortality among women in Turkey is breast cancer. Although several molecular and clinicopathological factors have been investigated in breast cancer prognosis, lymph node status is still the most important prognostic factor.[2] Lymph node-positive breast cancer is associated with poor prognosis as compared to node-negative tumors and also the number of involved lymph nodes is the most important and widely used independent prognostic factor in breast cancer.[3]

Breast cancer has a predilection to initially metastasize to the axillary lymph nodes, most commonly via the lymphatic system.[4] Members of the vascular endothelial growth factor (VEGF) family, VEGF-C and VEGF-D are thought to be the lymphangiogenic factors that stimulate lymphangiogenesis via activation of VEGF receptor (VEGFR)-3 specifically expressed on lymphatic endothelial cells.[5] Although the exact mechanisms and the role of lymphangiogenesis are unclear, VEGF-C and VEGF-D are believed to stimulate lymphangiogenesis. VEGFR-3 is expressed predominantly in the lymphatic endothelium and thus is considered to be a major regulator of lymphangiogenesis.

Staining of lymphatic endothelial cells by immunohistochemistry allows visualization of lymphatic vessels within and around tumors. D2-40/podoplanin is well known as one of the selective markers of lymphatic endothelial cells and therefore is used as a marker for identifying lymph vessels and detecting peri- and intra-tumoral lymphatic vessel density (LVD).

VEGF-C is considered to be a relatively specific lymphatic endothelial growth factor in normal tissues. Although VEGFR-3 is described as a predominantly lymphatic endothelial marker, Valtola et al. showed that VEGFR-3 is also very weakly expressed in the capillary endothelium of normal breast tissue.[6] The authors demonstrated that both intraductal and invasive cancer cells contain VEGF-C protein. VEGF-C, VEGF-D and their binding to the cognate receptor VEGFR-3 in the regulation of lymphangiogenesis is accepted, but their value for lymph node metastasis in breast cancer is controversial. In a number of studies, elevated VEGF-C expression has been reported in 30–40% of breast cancer. Although some studies have demonstrated that VEGF-C expression is significantly correlated with lymph node metastasis, some authors have found no correlation with lymphatic vessel invasion (LVI) or lymph node metastasis.[7,8]

The aim of this study was to investigate the possible relationships between VEGF-C, VEGF-D and VEGFR-3 expressions, LVD as determined by D2-40 and lymph node metastasis in primary invasive breast cancer. To the best of our knowledge, this is first study from Turkey which has evaluated the possible correlation between lymph node metastasis and the expression of VEGF-C, VEGF-D and VEGFR-3 and in breast cancer.

Material and methods

The study consisted of 80 female patients with invasive breast carcinoma (stage 1, stage 2 or stage 3), aged 31–80 years (mean 52 ± 14 years) who received primary surgical treatment by one surgical oncologist (A.E.) between 2008 and 2013 at the Department of Surgical Oncology, Ankara University Faculty of Medicine, and had sufficient paraffin-embedded tumor specimens. No patients had distant metastasis or received neoadjuvant chemotherapy or radiation therapy prior to surgery. The study was carried out with the approval of the Ethics Committee of Ankara University Faculty of Medicine (project no. 03-81-13) and a grant from the Scientific Research Project of Ankara University (project no. 13B3330015).

Immunohistochemical staining

For the 80 cases, the best paraffin block that showed the histological features of the tumor was selected; 4 µm sections were cut and stained using the streptavidin-biotin peroxidase method on a "Ventana automatic immunostaining device" (BenchMark XT Staining Module, Ventana Medical Systems) for VEGFR-3 (ABCAM), VEGF-C (ABCAM), VEGF-D (ABCAM) and D2-40 (DAKO) at 1 : 150, 1 : 20, 1 : 200 and 1 : 100 dilutions, respectively (Table 1). The antibody dilutions and sources are shown in Table 1. Appropriate control tissues were included in each staining run.

Cytoplasmic staining in invasive tumor cells was considered as positive in the assessment of VEGFR-3, VEGF-C and VEGF-D staining. The staining results were evaluated using the immunoreactive score (IRS) proposed by Remmele and Stegner (9) with a slight modification as follows: staining intensity (SI) × percentage of positive cells (PP). SI was defined as 0 = negative; 1 = weak; 2 = moderate; 3 = strong. PP was evaluated as 0 = negative; 1 = 1–50% positive cells; 2 = 51–100% positive cells. An IRS of 0 to 2 was considered as low-expression. IRSs from 3 to 6 were accepted as high-expression. In the D2-40 evaluation, the number of D2-40 stained lymphatic vessels were counted on 10 intra-tumoral (located within the tumor) and 10 peri-tumoral (located in the adjacent non-tumoral peripheral tissue) high power fields (HPFs).

Table 1. Source and technical characteristics of antibodies

Antibody	Clone	Dilution	Source
VEGFR-3	ab51496 mouse monoclonal	1 : 150	ABCAM
VEGF-C	ab9546 rabbit polyclonal	1 : 20	ABCAM
VEGF-D	ab155288 rabbit monoclonal	1 : 200	ABCAM
D2-40	M3619 mouse monoclonal	1 : 100	DAKO

Statistical analysis

Between-group comparisons were evaluated using the Mann-Whitney U-test or ANOVA test. The Lease Significant Difference (LSD) test was used for the post-hoc tests to determine which specific groups differ significantly from one another. Mean differences in peri- and intra-tumoral LVD and IRS counts were compared with the use of the paired t-test. The χ^2 test or Fisher's exact test were used to see if there was a relationship between 2 categorical variables. A p value < 0.05 was considered to be statistically significant. The statistical analysis was done using IBM SPSS Statistics Premium 22 V (License manager name: spss.hacettepe.edu.tr).

Results

VEGFR-3 expression was observed in tumor cells in 76 of 80 (95%) breast cancer patients. VEGF-C immunoreactivity was detectable in 67 (83.75%) patients. VEGF-D expression in tumor cells was found in 78 (97.5%) cases (Fig. 1).

Lymph node metastasis (N1 to N3) was positive in 51 (63.75%) patients. No significant association was found between VEGF-C or VEGFR-3 expression and lymph node status. However, a significant positive relationship between VEGF-D expression and the presence of N2 or N3 lymph node metastasis was observed (p < 0.05). As seen in Table 2, patients with N2 and N3 lymph node metastasis had a significantly higher IRS for VEGF-D.

Intra-tumoral LVD ranged from 0 to 3, peri-tumoral LVD ranged from 1 to 12 (Fig. 2). To correlate LVD and VEGF-D expressions, the patients were divided into 2 groups with regard to VEGF-D expression: low-level of VEGF-D expression and high-level of VEGF-D expression. There were 10 patients with low-expression of VEGF-D and 70 patients with high expression of VEGF-D. As shown in Table 3, patients with high-expression of VEGF-D were observed to have high levels of both peri- and intra-tumoral LVD compared to those with a low level of VEGF-D expression. In addition, VEGFR-3 expression in tumor cells was found to be significantly correlated with peri-tumoral LVD (p < 0.05), but not with intra-tumoral LVD (Table 3).

In addition to lymph node status, we evaluated the relationships between VEGF-C, VEGF-D and VEGFR-3 expressions and other clinicopathological variables. It was found that there were no significant correlation between VEGF-C, VEGF-D and VEGFR-3 expressions and the patient's age, tumor size, TNM stage or tumor grade (data not shown).

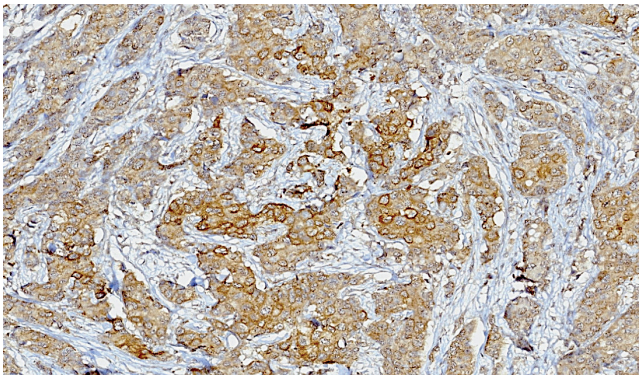

Fig. 1. Strong and diffuse VEGF-D staining in carcinoma cells, ×20

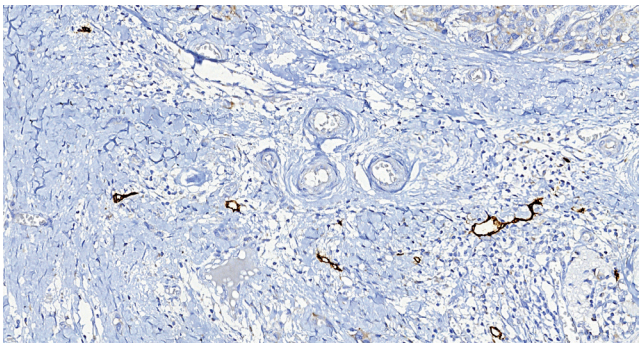

Fig. 2. Lymphatic vessels in peri-tumoral area, D2-40, ×10

Table 2. Relationship between lymph node status and mean VEGF-D IRS

Lymph node status	VEGF-D (IRS)	p value*
N0	2.69 ± 0.26	0.058
N1	3.23 ± 0.29	
N2	2.81 ± 0.59	
N3	4.11 ± 0.59	

***** ANOVA test was used ; p = 0.01 for N0 vs N3, and p = 0.031 for N2 vs N3, with LDS test

Table 3: Peri- and intra-tumoral lymphatic vessel density (LVD) according to the level of VEGF-D and VEGFR-3 expressions

Level of VEGF expressions	Peritumoral LVD	p-value*	Intratumoral LVD	p-value*
VEGF-D				
low expression (n = 10)	2.4 ± 0.8	0.002	0.8 ± 0.4	0.031
high expression (n = 70)	5.5 ± 3.1		1.29 ± 0.7	
VEGFR-3				
low expression (n = 12)	3.1 ± 2	0.014	1.2 ± 0.8	0.650
high expression (n = 68)	5.5 ± 3.1		1.3 ± 0.6	

* Mann-Whitney U test was used.

Discussion

It is known that breast cancer may spread via the lymphatic vessels into the axillary lymph nodes.[4] Lymphatic vessel invasion (LVI) has also been shown to significantly correlate with lymph node metastasis as well as sentinel and non-sentinel lymph node metastasis.[10] VEGF-C and VEGF-D are known as lymphangiogenic factors; both growth factors mediate their biological activity mainly by VEGFR-3.[11]

Although the relationships of VEGF-C and -D with lymph node metastasis are still a controversial issue, they are generally accepted to be significantly associated with the extent of lymph node metastasis in breast cancer.[12] Bando et al. evaluated intra-tumoral levels of VEGF-C and VEGFR-3 by using enzyme-linked immunosorbent assay in human breast cancer tissues and found that there was no significant prognostic value of VEGFR-3.[11] Recently, some authors have demonstrated that different molecular subtypes of breast cancer correlate with the expression of VEGF-C and/or VEGF-D.[13,14] Raica et al. investigated the differential of expressions VEGF-C, VEGFR-3 and D2-40 by immunohistochemistry to evaluate lymphangiogenesis and the lymphatic microvessel density in breast cancer patients.[13] They found that VEGF-C expression significantly correlated with lymph node metastasis, but not tumor grade. The authors showed the highest level of VEGF-C expression in HER2 and luminal types and the lowest expression in basal-like carcinoma. In contrast, Liu et al. showed that triple-negative breast cancer correlates with a high expression of VEGF-C and -D.[14]

Although some researchers have suggested that VEGF-C, but not VEGF-D, has an important role in lymphangiogenesis, others believe that VEGF-D is a predictor for lymphatic metastasis, and an independent prognostic factor.[15–19] The first group of researchers have suggested that VEGF-C promotes lymphangiogenesis and that tumor lymphangiogenesis in turn promotes lymphatic metastasis.[15,16,20] In the present study, we showed that VEGF-C, VEGF-D, and VEGFR-3 were expressed in a substantial percentage of breast carcinomas. VEGF-D expression was associated with lymph node metastasis, however there were no significant correlations between the expressions of VEGF-C and VEGFR-3 and lymph node status. In a study by Nakamura et al., LVD was evaluated by podoplanin immunostaining and the relationships between LVD and lymph node status as well as VEGF-C immunoreactivity and some clinicopathological parameters were analyzed.[20] The authors found that increased LVD was correlated with lymph node metastasis and VEGF-C expression. In their series, peri-tumoral lymph vessels were slightly higher in number when compared to intra-tumoral lymph vessels. Similarly, we have also observed that peri-tumoral lymph vessels were significantly higher than intra-tumoral lymphatic vessels in our study.

Contrary to the findings of the previous studies demonstrating that high expression of VEGF-C in human breast cancer is significantly correlated with lymph node metastasis and unfavorable prognosis, we have not observed any correlation of VEGF-C expression in tumor cells with lymph node status. In some studies, peri-tumoral LVD was found to be an independent risk factor for axillary lymph node metastasis.[10,21] In a recent study, VEGF-C and -D expressions were significantly associated with peri-tumoral LVD, as determined by D2-40 staining, but not intra-tumoral LVD in breast cancer.[22] The authors have found that peri-tumoral LVD was associated with lymph node metastasis, LVI and advanced tumor stage and thus they suggested that tumor derived VEGF-C and -D induce peri-tumoral lymphangiogenesis, which may be related to lymphatic invasion and metastatic spread. Mohammed et al. showed that high expressions of VEGF-A and -C, but not of VEGF-D, were associated with a higher LVD and lymph node metastasis.[16] In another study by Gu et al., LVD was found to be significantly associated with lymphatic metastasis and advanced tumor stage.[17] In their series, increased LVD was significantly correlated with high expressions of VEGF-C and -D. In their study, there was a close correlation between VEGF-D expression and lymphatic metastasis. In our study, VEGF-D expression was found to be significantly associated with both peri-tumoral and intra-tumoral LVD. There was also a significant correlation between VEGF-D expression and lymph node metastasis. We have observed that peri-tumoral, but not intra-tumoral, LVD was closely related to the high expression of VEGFR-3 in breast cancer cells.

In summary, our findings suggest that high level VEGF-D expression by breast cancer cells may induce lymphangiogenesis in the peri-tumoral and intra-tumoral region and thus contribute to a high peri- and intra-tumoral LVD, leading to lymphatic vessel invasion and metastatic spread to lymph nodes. According to the previous published studies and our present series, we can suggest that inhibition of VEGF-D expression to control peri- and intra-tumoral lymphangiogenesis may contribute to the development of novel therapeutic strategies for breast cancer treatment.[23]

References

1. Globocan 2012: Estimated cancer incidence, mortality and prevalence. http://globocan.iarc.fr/Pages/fact_sheets_population.aspx. Accessed 2015.
2. Untch M, Gerber B, Harbeck N, et al. 13th St. Gallen International Breast Cancer Conference 2013: Primary therapy of early breast cancer evidence, controversies, consensus-opinion of a German team of experts. (Zurich 2013). *Breast Care (Basel)* 2013;8:221–229.
3. Yang C, Liu F, Liu S, Li W, Zhai L, Ren M. Lymph node ratio. A new feature for defining risk category of node-positive breast cancer patients. *Int J Surg Pathol*. 2012;206:546–554.
4. Pepper MS. Lymphangiogenesis and tumor metastasis: More questions than answers. *Lymphology* 2000;33:144–147.
5. Auwera IV, Cao Y, Tille JC, et al. First international consensus on the methodology of lymphangiogenesis quantification in solid human tumours. *Br J Cancer*. 2006;95:1611–1625.

6. Valtola R, Salven P, Heikkila P, et al. VEGFR-3 and its ligand VEGF-C are associated with angiogenesis in breast cancer. *Am J Pathol*. 1999;154:1381–1390.
7. Koyama Y, Kaneko K, Akazawa C, et al. Vascular endothelial growth factor-C and vascular endothelial growth factor-D messenger RNA expression in breast cancer: Association with lymph node metastasis. *Clin Breast Cancer.* 2003;4:354–360.
8. Hoar FJ, Chaudhri S, Wadley MS, Stonelakc PS. Co-expression of vascular endothelial growth factor C (VEGF-C) and c-erbB2 in human breast carcinoma. *Eur J Cancer*. 2003;39:1698–1703.
9. Remmele W, Stegner HE. Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue. *Pathology*. 1987;8:138–140.
10. Ran S, Volk L, Hall K, Flister MJ. Lymphangiogenesis and lymphatic metastasis in breast cancer. *Pathophysiology*. 2010;17:229–251.
11. Bando H, Weich HA, Horiguchi S, et al. The association between vascular endothelial growth factor-C, its corresponding receptor, VEGFR-3, and prognosis in primary breast cancer: A study with 193 cases. *Oncology Reports*. 2006;15:653–659.
12. Skobe M, Hawighorst T, Jackson DG, et al. Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nat Med*. 2001;7:192–198.
13. Raica M, Cimpean AM, Ceausu R, Ribatti D. Lymphatic microvessel density, VEGF-C, and VEGFR-3 expression in different molecular types of breast cancer. *Anti Cancer Res*. 2011;31:1757–1764.
14. Liu HT, Ma R, Yang QF, Du G, Zhang CJ. Lymphangiogenic characteristics of triple-negative in node-negative breast cancer. *Int J Surg Pathol*. 2009;17:426–431.
15. Swartz MA, Skobe M. Lymphatic function, lymphangiogenesis, and cancer metastasis. *Microsc Res Tech*. 2001;55:92–99.
16. Mohammed RAA, Green A, El-Shikh S, et al. Prognostic significance of vascular endothelial growth factors -A, -C, and -D in breast cancer and their relationship with angio- and lymphangiogenesis. *Br J Cancer*. 2007;96:1092–1100.
17. Gu Y, Qi X, Guo S. Lymphangiogenesis induced by VEGF-C and VEGF-D promotes metastasis and a poor outcome in breast carcinoma: a retrospective study of 61 cases. *Clin Exp Metastasis*. 2008; 25:717–725.
18. Yasuoka H, Nakamura Y, Zuo H, et al. VEGF-D expression and lymph vessels play an important role for lymph node metastasis in papillary thyroid carcinoma. *Med Pathol*. 2005;18:1127–1133.
19. Yonemura Y, Endo Y, Tabata K, et al. Role of VEGF-C and VEGF-D in lymphangiogenesis in gastric cancer. *Int J Clin Oncol*. 2005;10:318–327.
20. Nakamura Y, Yasuoka H, Tsujimoto M, et al. Lymph vessel density correlates with nodal status, VEGF-C expression, and prognosis in breast cancer. *Breast Cancer Res Treat*. 2005;91:125–132.
21. Britto AV, Schenka AA, Moraes-Schenka NG, et al. Immunostaining with D2-40 improves evaluation of lymphovascular invasion, but may not predict sentinel lymph node status in early breast cancer. *BMC Cancer*. 2009;9:109.
22. Zhao YC, Ni XJ, Li Y, et al. Peritumoral lymphangiogenesis induced by vascular endothelial growth factor-C and D promotes lymph node metastasis in breast cancer patients. *World J Surg Oncol*. 2012; 10:165.
23. Dvorak HF, Weaver VM, Tlsty TD, Bergers G. Tumor microenvironment and progression. *J Surg Oncol*. 2011;103:468–474.

Dialysis vintage and cardiovascular injury as factors influencing long-term survival in peritoneal dialysis and hemodialysis

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Abstract

Background. Cardiovascular (CV) incidents are the major cause of mortality in maintenance dialysis (MD) patients undergoing peritoneal dialysis (PD) or hemodialysis (HD). CV injury indicators may be useful to investigate the dialysis modality influence on survival.

Objectives. The aim of this study was to compare selected laboratory and echocardiographic (ECHO) markers of CV injury in terms of dialysis vintage (DV), CV-related mortality and all-cause mortality.

Material and methods. The study involved 301 patients on HD (n = 301) and PD (n = 58), who were divided into subgroups according to DV. The subjects' medical histories included diabetes mellitus (DM), myocardial infarction (MI), stroke, CV deaths and deaths from non-CV causes. Their CV parameters were measured with ECHO for the left ventricle ejection fraction (EF), posterior wall (LVW) and interventricular septum (IVS). Serum analyses of cardiac troponin T (TnT) and N-terminal pro-brain natriuretic peptide (BNP) were also carried out.

Results. In the subgroup with a DV of 4 years, the PD and HD patients were of a similar age, and had similar mortality and morbidity rates and CV markers, except for thicker IVS in the HD patients.

Conclusions. Focusing on the data analysis based on mortality, and both laboratory and echocardiographic markers of cardiovascular injury, PD seems to be a more favorable method of dialysis. The advantage of PD was noted in subjects with a DV < 2 years. HD showed no outcome benefit over PD in longer DV.

Key words: hemodialysis, mortality, peritoneal dialysis, echocardiography, troponin T

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There is a lack of objective guidelines for initiating renal replacement therapy (RRT) with peritoneal dialysis (PD) or hemodialysis (HD) in patients with end-stage renal disease (ESRD) who develop uremic complications before receiving kidney transplantation. In routine practice, the choice of which dialysis modality to initiate is often based on the physicians' inclination, the patient's preferences, and any contraindications to one therapy or the other.[1–5] Clinical trials that randomize the dialysis modality initiated are currently lacking, since RRT qualification guidelines are based on social and ethical aspects.[6] Exploring the outcomes in current dialysis patient populations may therefore be worthwhile. Studies of that kind could analyze the effect of dialysis over different time periods, called the "dialysis vintage", as well as look for trends in patient survival, and could thus define mortality risk markers for different dialysis vintage periods.[6] As a result of the highly subjective criteria for dialysis initiation, populations undergoing maintenance HD or PD are significantly heterogeneous in terms of age, gender, ESRD background and comorbidities.[1,3] This means there is a need for reliable markers to objectively compare the safety of the different dialysis modalities. Recent epidemiological data confirm that cardiovascular (CV) incidents are the major contributors to the high mortality rate in the ESRD population.[7] Consequently, CV injury markers may be useful predictors in determining the effect of the 2 dialysis modalities on the recipient's survival.

Previous studies have provided evidence emphasizing the value of serum cardiac troponin T (cTnT) as a predictor of CV incidents and mortality.[8,9] N-terminal pro-brain natriuretic peptide (NT-proBNP) has been found to be another useful prognostic marker associated with mortality in dialysis patients.[9–11] The correlation between ESRD and development of CV disease (CVD) is well known. Renal failure affects both the structure and the function of the myocardium, which can be detected using echocardiography.[12–14] It would be beneficial to conduct a study comparing different CV variables and observing potential CV injury in patients on HD and PD.

The aim of the current study was to compare selected markers of CV injury in terms of their impact on morbidity and mortality within 2 cohorts of Polish patients undergoing maintenance dialysis with HD and PD respectively. After separating the dialysis patients into subgroups according to dialysis vintage, the outcomes of each group were analyzed.

Material and methods

A total of 359 Polish patients undergoing chronic dialysis during the period from 2010 to 2014 were followed-up for 24 months. They comprised 301 patients on thrice-weekly in-center HD (Group H) and 58 on home PD (Group P). The exclusion criteria were a lack of written consent, RRT vintage lower than 6 weeks and age below 18 years old. Subjects with a history of dialysis modality conversion before qualification to the study were also excluded. The patients in this study provided informed written consent, and the Poznan University of Medical Sciences Ethics Committee approved the study.

The patients' history included the ESRD background, diabetes mellitus (DM), myocardial infarction (MI) during follow-up, stroke during follow-up and end-point (death, renal transplantation, conversion to another type of dialysis or termination of dialysis). The cause of death was specified as CV (including sudden cardiac arrest, acute MI, venous thromboembolism and consequences of heart failure) or non-CV. Each of the 2 dialysis modality groups were divided into 3 subgroups depending on the dialysis vintage: a) HD and PD patients in subgroups HA and PA received dialysis for < 104 weeks (< 2 years); b) patients included in subgroups HB and PB remained on dialysis treatment with HD and PD respectively for 104–208 weeks (2–4 years); and c) subgroups HC and PC included patients whose dialysis vintage on HD and PD respectively exceeded 208 weeks (> 4 years).

A number of procedures were performed on each patient at the beginning of observation (for HD patients before the midweek dialysis session). Blood samples were obtained before dialysis to analyze CV injury markers cTnT (using the Elecsys®/cobas e™ cTnT fourth-generation assay, Roche Diagnostics, Basel, Switzerland) and NT-proBNP. Transthoracic echocardiography was performed following current guidelines to assess left ventricle parameters: ejection fraction, posterior wall (LVW) and interventricular septum (IVS) thickness.[15] The statistical analysis was carried out using STATISTICA 12.5 software (StatSoft, Tulsa, USA). All correlations were calculated using the Spearman correlation coefficient. The comparisons of the HD and PD group variables were performed using either the Mann-Whitney or the t-test, depending on normality. A survival analysis was conducted using Kaplan-Meier curves. Logistic regression was used for a multivariable analysis of markers affecting mortality.

Results

Characteristics of the HD and PD study participants

A summary of patient characteristics is shown in Table 1. The 301 HD patients studied were significantly older than the 58 PD patients (64 ± 15 vs 56 ± 17 years; $p < 0.001$), and they were more often men (63% vs 48%; $p < 0.05$). The mean dialysis vintage was longer for HD patients than for PD patients (264 ± 216 vs 135 ± 96 weeks; $p < 0.001$). Globally there was no significant difference in the incidence of DM, MI or stroke. The gross mortality, expressed as a percentage, was higher for HD than for PD

Table 1. Comparison of groups on hemodialysis (H) and peritoneal dialysis (P)

Parameter	H	P	p-value
Age, years	64 ± 15	56 ± 17	< 0.001
Patients, n	301	58	
Males/females, n	190/111	28/30	< 0.05
Dialysis vintage, weeks	264 ± 216	135 ± 96	< 0.001
Comorbidity and deaths			
DM, n	95	19	0.99
MI, n	48	5	0.13
Stroke, n	26	2	0.16
All-cause deaths, n	89	6	< 0.01
Cardiovascular-related deaths, n	44	5	< 0.001
Tx, n	23 (7.6%)	9 (15.5%)	< 0.05
Predictors investigated			
BNP, pg/mL	12192 ± 12873	6571 ± 10203	< 0.001
cTnT, ng/mL	75.5 ± 84.4	71.8 ± 99.1	< 0.05
IVS, mm	14.1 ± 2.8	12.2 ± 2.1	< 0.001
LVW, mm	13.4 ± 2.3	12.0 ± 2.3	< 0.01
EF, %	57.9 ± 11.2	54.8 ± 11.6	0.15

Data are presented as mean ± standard deviation; BNP – N-terminal pro-brain natriuretic peptide; cTnT – cardiac troponin T; DM – diabetes mellitus; DN – diabetic nephropathy; EF – ejection fraction of left ventricle; GN – glomerulonephritis; HT – hypertension; IVS – interventricular septum; LVW – left ventricle posterior wall; MI – myocardial infarction; Tx – kidney transplantation.

patients (29.6% vs 10.3%; p < 0.001), while CV mortality was comparable for HD and PD (Table 2). The rate of transplantation was higher in PD than HD patients (15.5% vs 7.6%; p < 0.05). The mean concentrations of cardiac markers were substantially higher in HD than PD patients: For NT-proBNP it was 12192 ± 12873 vs 6571 ± 10203 pg/mL (p < 0.001); for cTnT it was 75.5 ± 84.4 vs 71.8 ± 99.1 ng/mL (p < 0.05). Echocardiography demonstrated that both the mean IVS (14.1 ± 2.8 vs 12.2 ± 2.1 mm; p < 0.001) and the LVW (13.4 ± 2.3 vs 12.0 ± 2.3 mm; p < 0.01) were thicker in HD than PD patients, while the 2 groups did not differ with regard to EF. Selected correlations are presented in Table 3.

The Kaplan-Meier curves illustrate that DM was a main factor shortening survival time, both in patients on HD (Fig. 1a; p < 0.05) and those on PD (Fig. 1b; p < 0.05). Age > 65 years appeared to be a negative prognostic factor only for the HD patients (Fig. 1c; p < 0.01), not for PD patients (Fig. 1d).

There are numerous risk factors and potential predictors of dialysis recipients' mortality and their influence is not independent. For precise assessment of each parameter's influence in vivo, is necessary to combine them in models. Multivariable logistic regression analysis was introduced to create models including statistically significant identify markers affecting all-cause and CV mortal-

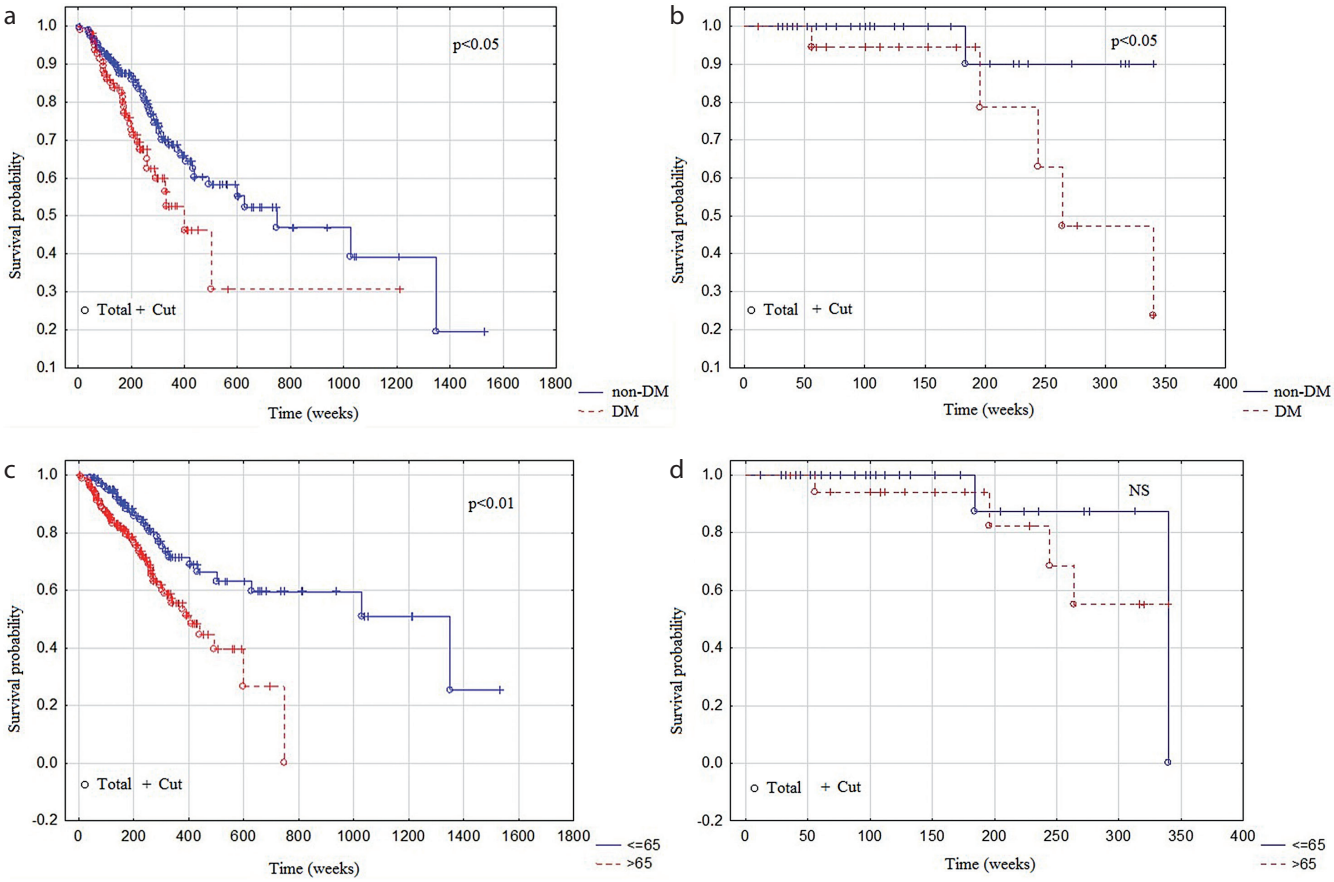

Fig. 1. Kaplan-Meier survival probability curves; a) HD survival with/without DM; b) PD survival with/without DM; c) age-dependend survival on HD; d) age-dependend survival on PD; DM – diabetes mellitus; HD – hemodialysis; PD – peritoneal dialysis.

Table 2. All-cause mortality and CV-related mortality in terms of dialysis modality

Parameter	Survived entire follow-up period	Died during follow-up period	All
Dialysis modality		all causes	
HD (n)	212	89	301
% of the HD subgroup	70.4%	29.6%[a]	100%
PD (n)	52	6	58
% of the PD subgroup	89.7%	10.3%[a]	100%
Dialysis modality		CV-related	
HD (n)	257	44	301
% of the HD subgroup	85.4%	14.6%[a]	100%
PD (n)	53	5	58
% of the PD subgroup	91.4%	8.6%[a]	100%

[a] p < 0.001; CV – cardiovascular; HD – hemodialysis; PD – peritoneal dialysis.

Table 3. Correlations between selected factors in HD and PD patients

Dialysis/parameter	Parameter	r	p-value
HD + PD			
Death	age	0.19	< 0.001
Death	DM	0.12	< 0.05
Age	DM	0.20	< 0.05
HD			
MI	death	0.10	0.08
BNP	death	0.22	< 0.001
cTnT	death	0.23	< 0.001
cTnT	MI	0.06	0.3
cTnT	LVW	0.47	< 0.001
BNP	LVW	0.41	< 0.001
Death	DM	0.08	0.1
Age	DM	0.15	< 0.05
PD			
MI	death	0.90	< 0.001
cTnT	death	0.29	< 0.05
BNP	death	0.16	0.2
cTnT	MI	0.25	0.06
cTnT	LVW	–0.14	0.4
BNP	LVW	–0.05	0.8
Death	DM	0.37	< 0.05
Age	DM	0.45	< 0.05

BNP – N-terminal pro-brain natriuretic peptide; cTnT – cardiac troponin T; DM – diabetes mellitus; HD – hemodialysis; IVS – interventricular septum; LVW – left ventricle posterior wall; MI – myocardial infarction; PD – peritoneal dialysis.

ity of the patients on HD; a corresponding model for PD was not possible due to the low mortality rate in the PD group. Both of the models presented for HD revealed that an increase in cTnT levels, dialysis vintage < 104 weeks and age > 65 years were connected with a higher risk of death (Table 4; p < 0.001). None of the models including markers of CV injury other than cTnT as variables appeared significant.

Table 4. Logistic regression multivariable analysis of all-cause and CV-related mortality risk factors among the HD patients

Covariate	OR	95% CI	p-value
All-cause deaths			**< 0.001**
TnT	4.25	2.30–7.85	< 0.001
HD vintage	0.45	0.31–0.66	< 0.001
Age	2.18	1.25–3.81	< 0.01
CV-related deaths			**< 0.001**
TnT	3.61	1.75–7.44	< 0.001
HD vintage	0.49	0.31–0.76	< 0.01
Age	2.80	1.33–5.90	< 0.01

CI – confidence interval; cTnT – cardiac troponin T; HD – hemodialysis; OR – odds ratio.

A. Dialysis vintage < 2 years: Subgroup HA vs subgroup PA

In the study group, 13.6% of the HD patients and 48.3% of the PD patients had been on dialysis for less than 24 months (p < 0.001). As shown in Table 5, the

Table 5. Comparison of subgroups on hemodialysis (HA) and peritoneal dialysis (PA) for < 104 weeks (< 2 years)

Parameter	HA	PA	p-value
Age, years	65.5 ± 18.5	50.9 ± 17.1	< 0.01
Patients, n	41	28	
Males/females, n	26/15	12/16	0.15
Dialysis vintage, weeks	66.4 ± 26.6	57.0 ± 26.7	0.11
Comorbidity and deaths			
DM, n	15	7	0.22
MI, n	8	1	0.05
Stroke, n	2	1	0.76
All-cause deaths, n	28	1	< 0.001
Cardiovascular-related deaths, n	15	1	< 0.01
Tx, n	6	7	0.29
Predictors investigated			
BNP, pg/mL	13967 ± 13449	4464 ± 7643	< 0.01
cTnT, ng/mL	70.1 ± 56.0	40.5 ± 50.0	< 0.001
IVS, mm	12.7 ± 3.8	12.1 ± 2.5	0.73
LVW, mm	13.3 ± 3.2	11.8 ± 2.7	0.37
EF, %	61.0 ± 9.5	55.3 ± 11.4	0.38

Data are presented as mean ± standard deviation; BNP – N-terminal pro-brain natriuretic peptide; cTnT – cardiac troponin T; DM – diabetes mellitus; DN – diabetic nephropathy; EF – ejection fraction of left ventricle; GN – glomerulonephritis; HT – hypertension; IVS – interventricular septum; LVW – left ventricle posterior wall; MI – myocardial infarction; Tx – kidney transplantation.

HA patients were significantly older than the PA patients (65.5 ± 18.5 vs 50.9 ± 17.1 years; p < 0.01) while the gender distribution in the 2 subgroups was comparable. There was no significant difference noted in the mean dialysis vintage, the presence of DM or the incidence of stroke. A substantially higher incidence of MI (8 vs 1; p < 0.05) and higher mortality rate (28 [15 CV] vs 1; p < 0.001) were recorded in group HA in comparison to group PA. In the HA group, 6 patients received kidney transplants, and kidney function improved in 2 subjects with no further dialysis requirement. In the PA group, 7 patients underwent transplantation, while 2 converted to HD. The mean serum concentrations of CV markers were higher in the HA group compared to the PA group: For NT-proBNP they were 13967 ± 13449 vs 4464 ± 7643 pg/mL (p < 0.01), and for cTnT they were 70.07 ± 55.98 vs 40.54 ± 49.95 ng/mL (p < 0.001). The mean values of echocardiographic parameters did not differ between these 2 subgroups.

B. Dialysis vintage 2–4 years: Subgroup HB vs subgroup PB

A similar proportion of HD and PD patients had a dialysis vintage of 2–4 years. The mean age, gender distribution and dialysis vintage were similar in the HB and PB subgroups (Table 6). Additionally, the incidence of MI, stroke, DM and mortality rate did not differ significantly in the HB and PB groups. In the HB group, 10 patients received kidney transplants, and 1 patient was switched to PD. In the PB group, 1 patient underwent a transplant and 5 required the conversion to HD. No differences were observed between these 2 subgroups with regard to the mean serum values of CV markers NT-proBNP and cTnT or echocardiographic indicators IVS, LVW, EF.

C. Dialysis vintage > 4 years: Subgroup HC vs subgroup PC

Among the patients with a dialysis vintage of > 4 years, there was a higher proportion of HD than PD patients (49.5% vs 22.4%; p < 0.001). The mean age and gender distribution in the HC and PC subgroups were comparable (Table 7). The mean dialysis vintage was slightly higher in the HC subgroup than in the PC subgroup (404 ± 232 vs 286 ± 45 weeks; p = 0.06). There was no significant difference in the incidence of MI, stroke or the presence of DM. The death rate was also comparable in these 2 subgroups. Within the HC subgroup, 7 patients received kidney transplants, while 1 patient was transferred to another HD center and was lost to further follow-up. One PC patient received a transplant and 3 required conversion to HD. The mean concentrations of NT-proBNP and cTnT were comparable in subgroups HC and PC. Among the echo-

Table 6. Comparison of subgroups on hemodialysis (HB) and peritoneal dialysis (PB) for 104–208 weeks (2–4 years)

Parameter	HB	PB	p-value
Age, years	62.7 ± 16.3	57.8 ± 17.3	0.24
Patients, n	111	17	
Males/females, n	66/45	8/9	0.34
Dialysis vintage, weeks	149.7 ± 29.2	148.9 ± 33.2	0.78
Comorbidity and deaths			
DM, n	33	7	0.43
MI, n	16	1	0.31
Stroke, n	8	1	0.81
All-cause deaths, n	23	2	0.39
Cardiovascular-related deaths, n	12	1	0.54
Tx, n	10	1	0.67
Predictors investigated			
BNP, pg/mL	9164 ± 11477	8012 ± 12156	0.24
cTnT, ng/mL	62.3 ± 65.7	103.7 ± 149.1	0.97
IVS, mm	12.3 ± 2.1	12.4 ± 1.8	0.57
LVW, mm	12.4 ± 1.8	12.25 ± 1.8	0.78
EF, %	56.9 ± 10.2	52.17 ± 13.5	0.45

Data are presented as mean ± standard deviation; BNP – N-terminal pro-brain natriuretic peptide; cTnT – cardiac troponin T; DM – diabetes mellitus; DN – diabetic nephropathy; EF – ejection fraction of left ventricle; GN – glomerulonephritis; HT – hypertension; IVS – interventricular septum; LVW – left ventricle posterior wall; MI – myocardial infarction; Tx – kidney transplantation.

Table 7. Comparison of subgroups on hemodialysis (HC) and peritoneal dialysis (PC) for > 208 weeks (> 4 years)

Parameter	HC	PC	p-value
Age, years	64.4 ± 13.8	63.7 ± 13.1	0.90
Patients, n	149	13	
Males/females, n	98/51	5/8	0.76
Dialysis vintage, weeks	403.7 ± 232.0	285.5 ± 44.5	0.06
Comorbidity and deaths			
DM, n	47	5	0.62
MI, n	24	3	0.53
Stroke, n	16	0	0.22
All-cause deaths, n	38	3	0.85
Cardiovascular-related deaths, n	17	3	0.22
Tx, n	7	1	0.64
Predictors investigated			
BNP, pg/mL	13973 ± 13354	9337 ± 12177	0.22
cTnT, ng/mL	86.6 ± 100.6	97.3 ± 83.7	0.30
IVS, mm	14.7 ± 2.8	12.2 ± 1.6	< 0.01
LVW, mm	13.7 ± 2.3	12.3 ± 1.8	0.11
EF, %	58.1 ± 11.8	56.9 ± 10.3	0.65

Data are presented as mean ± standard deviation; BNP – N-terminal pro-brain natriuretic peptide; cTnT – cardiac troponin T; DM – diabetes mellitus; DN – diabetic nephropathy; EF – ejection fraction of left ventricle; GN – glomerulonephritis; HT – hypertension; IVS – interventricular septum; LVW – left ventricle posterior wall; MI – myocardial infarction; Tx – kidney transplantation.

cardiographic parameters analyzed, only IVS differed significantly: It was thicker in subgroup HC (14.71 ± 2.75 vs 12.22 ± 1.56 mm; $p < 0.01$).

Discussion

In the current study, characteristics of HD and PD patients treated over different periods of time (dialysis vintage) were compared within each vintage group. Although the HD population studied was significantly older than the PD patients, it did not present a higher incidence of DM, MI or stroke. A natural, increasing proportion of HD patients with a longer dialysis vintage was observed, possibly due to the time-dependent limitation of the peritoneum as a dialytic membrane resulting in a need for conversion from PD to HD.[16,17] Another explanation for the increasing proportion of HD patients with a longer dialysis vintage is the higher incidence of renal transplantation in PD patients.

A higher mortality rate was observed on HD as compared to PD, but this was limited to patients on dialysis for less than 2 years. There are numerous causes that could be responsible for the higher early mortality observed among HD patients. The first possible cause is the significantly lower mean age of the patients undergoing PD. As expected, age and death rate were correlated (Table 3), and the influence of age on death rate was illustrated by Kaplan-Meier curves (Fig. 1c). This result is due to this study's aim of comparing patients in terms of dialysis vintage. PD is often more likely in younger ESRD patients because it offers them more independence as compared to HD. Another probable explanation is that the start of PD is usually characterized by less urgency. Therefore, dialysis initiation with PD is mainly reserved for patients under pre-dialytic care who are in stable condition and receiving conservative treatment.[18] A 3rd hypothesis for the higher mortality rate in HD during the first 2 years of therapy may be related to psychosocial and economic factors related to HD and PD: Patients who choose PD seem to be more active, have lower rate of comorbidities and benefit from having better family support.[19,20]

Although CV-related mortality compared to total all-cause mortality was in fact higher in PD, overall mortality rates – both CV-related and all-cause mortality – were higher in HD. Signs of non-specific CV injury, reflected by both laboratory markers (cTnT, NT-proBNP) and echocardiographic indicators (IVS, LVW) were more prominent in HD than in PD patients. Furthermore, increases in both cTnT and NT-proBNP showed a positive correlation with the mortality rate in HD patients, while in PD patients only cTnT correlated with mortality. Increases in both cTnT and NT-proBNP were, however, connected with LV wall hypertrophy in the HD population. Interestingly, the laboratory CV injury markers analyzed appeared to be predictors of overall all-cause mortality, but not specifically of CV-related mortality. These results indicate that dialysis and ESRD may induce specific CV injuries that lead to increased mortality which are not reflected in myocardial hypertrophy or in EF decrease. It can only be suggested that these 2 echocardiographic findings may illustrate a mechanism of myocardial adaptation to an increased CV burden, rather than a direct cause of poor outcomes.

The results outlined above appear to show that PD has an advantage in terms of patient outcomes. However, due to the lack of randomized trials comparing HD and PD in the ESRD population,an objective comparison of both modalities is difficult.[6] As a possible alternate solution, propensity score-stratified (PS) models that serve as an equivalent of randomization have been proposed. A study by Liem et al. confronted a PS model with a multivariable-adjusted (MV) model in survival estimation.[21] The same variables were included in both models. With the PS model, covariate balance between the groups was achieved. On the other hand, the features from the PS model were entered into the MV model as covariates. Eventually, the hazard ratios for mortality in both models appeared to be identical: 0.99 vs 0.97 respectively for the MV and PS models.[21] In the current study, a MV logistic regression model was introduced to estimate the influence of the variables analyzed on the differences between the subgroups undergoing HD or PD in order to assess their influence on the disproportionate outcomes. A strong disadvantage of both the PS and MV models is their low utility for small sample sizes as in the current study. Furthermore, due to the low mortality rate in the PD study group, a significant logistic regression MV model could not be created for this population. In the HD population, an increase in cTnT, dialysis vintage < 104 weeks and age > 65 years were associated with increased overall and CV mortality.

Previous studies – either prospective, often performed in small groups and lacking the statistical power of large cohort analyses, or retrospective, which rely on a large number of patient samples in national or dialysis center registries – have not delivered definitive proof of one modality being superior over the other.[22–28] Research based on PD and HD groups adjusted for demographic factors and comorbidities has commonly warned of increased global mortality in PD populations.[22,25,26] Nevertheless, after the year 2000, improvements in dialysis methods and supportive therapies appear to have resulted in a more substantial decrease of mortality rates among PD patients compared to HD patients.[7,20,27,29,31,32] Hence, in order to determine the advantages of each modality, there must be an up-to-date analysis of overall outcomes taking influential factors into account. Differences between HD and PD long-term survival have been reported to depend on the dialysis vintage. Previous studies have announced different cutoff points that are favorable for PD as an initial dialysis modality.[7,24,26,30,33] The results of the current study

showed a significantly better prognosis for both survival and the probability of renal transplantation in patients on maintenance PD for less than 24 months. A favorable outcome for HD was not observed either in the medium term or in the longest dialysis vintage group.

Most recent studies reveal a survival advantage for PD patients younger than 65 years old, non-diabetics and those with only one comorbidity.[20,30,34,35] According to the current results and those of other research groups, initiating dialysis with PD seems to be more favorable in general. The exception is that a longer dialysis vintage reduces the advantage of PD.[20,30–32,34,35] Nonetheless, without selecting specific patient characteristics, no survival benefit was proved for any of the HD vintage subgroups. A prospective comparison of survival among patients converted from PD – after the favorable vintage of up to 2 years – to HD would be required. A crucial variable that was not considered by this study is a patient's quality of life on the preferred dialysis modality. Its influence on survival is immeasurable using non-randomized comparative analyses of the advantages of HD and PD.

This prospective observational study comparing the effectiveness of dialysis modalities carries several limitations. First, due to limitations arising from the ESRD population, it does not have the quality of a randomized trial. Patients who had initiated RRT in a different modality were excluded; however, patients with an unplanned switch to HD were qualified. Moreover, the analysis did not take into account comorbidities other than those mentioned, or changes in a patient's health status over time. Another source of bias emerged from demographic differences between the populations compared. Nevertheless, the patients' demographic and psychosocial variations play a major role in modality selection; therefore a comparison of heterogeneous populations of ESRD patients on different dialysis modalities seems reasonable. To evaluate the influence of demographic variables on survival, a multivariable analysis was performed; however, its value is limited by the relatively low number of patients in the PD group and – as a result – its low mortality rate. The data presented in the study do not include vascular access in HD patients or PD regimens.

A definite advantage of this study is that it represents a contemporary prospective multifactorial comparison of a fairly large Polish cohort of patients undergoing maintenance dialysis. The qualification criteria excluded patients who had converted from one dialysis modality to another, to eliminate this source of bias. Focusing on the frequency of CV-related mortality in the ESRD population, the patients on each modality were divided into subgroups according to their dialysis vintage, and (as described above) analyzed in terms of selected co-morbidities, CV incidents, CV-related mortality and all-cause mortality. Laboratory markers and echocardiographic indicators of CV injury were evaluated as potential predictors of a poor prognosis.

In summary, in terms of mortality, as well as laboratory markers and echocardiographic indicators of cardiovascular injury, PD seems a more favorable method of dialysis. Nevertheless, PD recipients were younger and had more often received early pre-dialysis nephrological care. The influence of these factors on the parameters measured cannot be precisely calculated. Therefore, the advantage of PD cannot be clearly confirmed. Moreover, this advantage of PD was observed only in patients on dialysis for less than 2 years. With longer dialysis vintages, the laboratory and clinical advantages of PD were no longer present. However, even with longer dialysis vintages, HD showed no benefit with regard to outcomes. In addition to individual preferences of patients, PD seems to be the dialysis method of choice for candidates anticipating early kidney transplantation. Nevertheless, a more reliable analysis of the benefits of each modality would require an evaluation of the patients' quality of life.

References

1. Oliver MJ, Garg AX, Blake PG, et al. Impact of contraindications, barriers to self-care and support on incident peritoneal dialysis utilization. *Nephrol Dial Transplant*. 2010;25:2737–2744.
2. Blake PG, Quinn RR, Oliver MJ, et al. Peritoneal dialysis and the process of modality selection. *Perit Dial Int*. 2013;33:233–241.
3. Stack AG. Determinants of modality selection among incident US dialysis patients: Results from a national study. *J Am Soc Nephrol*. 2002;13:1279–1287.
4. Wankowicz Z. Peritoneal dialysis and its role in the demography and epidemiology of chronic kidney disease. *Pol Arch Med Wewn*. 2009;119:810–814.
5. Bargman JM. Timing of initiation of RRT and modality selection. *Clin J Am Soc Nephrol*. 2015;10:1072–1077.
6. Korevaar JC, Feith GW, Dekker FW, et al. Effect of starting with hemodialysis compared with peritoneal dialysis in patients new on dialysis treatment: A randomized controlled trial. *Kidney Int*. 2003; 64:2222–2228.
7. Collins AJ, Foley RN, Chavers B, et al. 2013 USRDS Annual data report: Atlas of chronic kidney disease and end-stage renal disease in the United States. *Am J Kidney Dis*. 2014;59:e73–e82.
8. Khan NA, Hemmelgarn BR, Tonelli M, et al. Prognostic value of troponin T and I among asymptomatic patients with end-stage renal disease: A meta-analysis. *Circulation*. 2005;112:3088–3096.
9. McGill D, Talaulikar G, Potter JM, et al. Over time, high-sensitivity TnT replaces NT-proBNP as the most powerful predictor of death in patients with dialysis-dependent chronic renal failure. *Clin Chim Acta*. 2010;411:936–939.
10. Hickman PE, McGill DA, Potter JM, et al. Multiple biomarkers including cardiac troponins T and I measured by high-sensitivity assays, as predictors of long-term mortality in patients with chronic renal failure who underwent dialysis. *Am J Cardiol*. 2015;115:1601–1606.
11. Schwermer K, Hoppe K, Radziszewska D, et. al. N-terminal pro-B-type natriuretic peptide as a marker of hypervolemia and predictor of increased mortality in patients on hemodialysis. *Pol Arch Med Wewn*. 2015;125:560–569.
12. Rebic DR, Rasic SR, Dervisevic MD, et al. Alteration of cardiovascular structure and function in patients undergoing peritoneal dialysis. *Cardiorenal Med*. 2015;5:135–144.
13. Koo HM, Doh FM, Kim CH, et al. Changes in echocardiographic parameters according to the rate of residual renal function decline in incident peritoneal dialysis patients. *Medicine (Baltimore)*. 2015; 94(7):e427. doi:10.1097/MD.0000000000000427.
14. Franczyk-Skora B, Gluba A, Olszewski R, et al. Heart function disturbances in chronic kidney disease: Echocardiographic indices. *Arch Med Sci*. 2014;10:1109–1116.
15. Lang RM, Bierig M, Devereux RB, et al. Recommendations for chamber quantification. *Eur J Echocardiogr*. 2006;7:79–108.

16. Guo, A. and S. Mujais. Patient and technique survival on peritoneal dialysis in the United States: Evaluation in large incident cohorts. *Kidney Int Suppl*. 2003;88:3–12.
17. Woodrow G, Turney JH, Brownjohn AM. Technique failure in peritoneal dialysis and its impact on patient survival. *Perit Dial Int*. 1997; 17:360–364.
18. Ivarsen P, Povlsen JV. Can peritoneal dialysis be applied for unplanned initiation of chronic dialysis? *Nephrol Dial Transplant*. 2014;29:2201–2206.
19. Winkelmayer WC, Glynn RJ, Levin R, et al. Late referral and modality choice in end-stage renal disease. *Kidney Int*. 2001;60:1547–1554.
20. van de Luijtgaarden MW, Jager KJ, Segelmark M, et al. Trends in dialysis modality choice and related patient survival in the ERA-EDTA: Registry over a 20-year period. *Nephrol Dial Transplant*. 2016; 31:120–128.
21. Liem YS, Wong JB, Hunink MG, et al. Propensity scores in the presence of effect modification: A case study using the comparison of mortality on hemodialysis versus peritoneal dialysis. *Emerg Themes Epidemiol*. 2010;7(1):1. doi: 10.1186/1742-7622-7-17:1.
22. Jaar BG, Coresh J, Plantinga LC, et al. Comparing the risk for death with peritoneal dialysis and hemodialysis in a national cohort of patients with chronic kidney disease. *Ann Intern Med*. 2005;143:174–183.
23. Murphy SW, Foley RN, Barrett BJ. Comparative mortality of hemodialysis and peritoneal dialysis in Canada. *Kidney Int*. 2000;57:1720–1726.
24. Termorshuizen F, Korevaar JC, Dekker FW, et al. Hemodialysis and peritoneal dialysis: Comparison of adjusted mortality rates according to the duration of dialysis: Analysis of the Netherlands cooperative study on the adequacy of dialysis 2. *J Am Soc Nephrol*. 2003; 14:2851–2860.
25. Ganesh SK, Hulbert-Shearon T, Port FK, et al. Mortality differences by dialysis modality among incident ESRD patients with and without coronary artery disease. *J Am Soc Nephrol*. 2003;14:415–424.
26. Stack AG, Molony DA, Rahman NS, et al. Impact of dialysis modality on survival of new ESRD patients with congestive heart failure in the United States. *Kidney Int*. 2003;64:1071–1079.
27. Vonesh EF, Snyder JJ, Foley RN, Collins AJ. The differential impact of risk factors on mortality in hemodialysis and peritoneal dialysis. *Kidney Int*. 2004;66:2389–2401.
28. Heaf JG, Lokkegaard H, Madsen M. Initial survival advantage of peritoneal dialysis relative to haemodialysis. *Nephrol Dial Transplant*. 2002;17:112–117.
29. Perl J, Wald R, Bargman JM, et al. Changes in patient and technique survival over time among incident peritoneal dialysis patients in Canada. *Clin J Am Soc Nephrol*. 2012;7:1145–1154.
30. Yeates K, Zhu N, Vonesh E, et al. Hemodialysis and peritoneal dialysis are associated with similar outcomes for end-stage renal disease treatment in Canada. *Nephrol Dial Transplant*. 2012;27:3568–3575.
31. Heaf JG, Wehberg S. Relative survival of peritoneal dialysis and haemodialysis patients: Effect of cohort and mode of dialysis initiation. *PLoS One*. 2014; 9(3):e90119. doi: 10.1371/journal.pone.0090119.
32. Kumar VA, Sidell MA, Jones JP, Vonesh EF. Survival of propensity matched incident peritoneal and hemodialysis patients in a United States health care system. *Kidney Int*. 2014;86:1016–1022.
33. McDonald SP, Marshall MR, Johnson DW, Polkinghorne KR. Relationship between dialysis modality and mortality. *J Am Soc Nephrol*. 2009;20:155–163.
34. Weinhandl ED, Foley RN, Gilbertson DT, et al. Propensity-matched mortality comparison of incident hemodialysis and peritoneal dialysis patients. *J Am Soc Nephrol*. 2010;21:499–506.
35. Mehrotra R, Chiu YW, Kalantar-Zadeh K, et al. Similar outcomes with hemodialysis and peritoneal dialysis in patients with end-stage renal disease. *Arch Intern Med*. 2011;171:110–118.

Ductal carcinomas in situ and invasive cancers detected on screening mammography: Cost-effectiveness of initial and subsequent rounds of population-based program 2007–2014

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Abstract

Background. Potential benefits of screening need to be carefully balanced against the financial burden for the national health care system.

Objectives. To assess the cost-effectiveness of population-based mammographic screening in the 3-million region of Lower Silesia (Poland) after initial and 3 subsequent rounds.

Material and methods. Data was collected in a prospective manner using the databases of the official computer system for the monitoring of prophylaxis programs (SIMP), National Health Fund (Lower Silesia Regional Branch) and the Lower Silesia Cancer Registry. The expenses from each analyzed year were obtained from the Regional Coordinating Center for Screening Programs. The number of screen-detected and pathologically proven invasive and ductal in situ cancers was calculated. Costs of cancer detection were measured, converted into US dollars (USD), and expressed in 2015 USD using the comparison of purchasing power of money calculated with the Consumer Price Index.

Results. The total expense for the screening program in the initial round (2007–2008), first (2009–2010), second (2011–2012) and third (2013–2014) subsequent rounds was 4 732 383, 6 043 509, 6 484 834, and 5 900 793 USD whereas the number of cancer detected was 1049, 987, 1312, and 1070. The cost-effectiveness ratio obtained in the program for each year was 4511, 6123, 4943, and 5515 USD per cancer found. The average cost of breast cancer detection in screening program in the region of Lower Silesia in years 2007–2014 was 5243 USD.

Conclusions. The low cost of breast cancer detection in mammographic screening program makes it applicable for the health care systems in emerging economies.

Key words: breast cancer, mammographic screening, cost-effectiveness

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Introduction

Despite important advances in therapy and translational research, strategies for breast cancer control are geared towards early detection of the disease.[1–3] In high-income economies, such as US or Western European Countries, mammographic screening has been widely implemented, leading to various improvements in breast cancer treatment outcomes.[3–6] However, cost-effectiveness of organized and centralized screening has to be taken into account when a nation-wide program is introduced, in particular in emerging countries. Although Poland is ranked by the World Bank as belonging to high-income countries (http://data.worldbank.org/country), the gross national income (GNI) per capita (13 730 USD) remains considerably lower than in Western Europe, and is just slightly above the borderline value (12 616 USD), which results in strongly limited health care budget. There is mixed data regarding the economic attractiveness of mammographic screening in low- and middle-income countries. The evidence base is still too small to generalize these findings and draw significant conclusions.[3]

Lower Silesia is a region with 3 million inhabitants in southwest Poland. Since the introduction of mammographic screening, approximately 60 000 women have been screened each year. During the initial (2007–2008) and first subsequent (2009–2010) round of screening the technical recall rate was 0.27% and 0.1%, whereas further assessment rate 6.2% and 4.5%, respectively. The cancer detection rate (both invasive and ductal in situ) was 6.6/1000 and 5.5/1000 while cancer detection rate, expressed as a multiple of the expected breast cancer incidence rate, was 3.8 and 3.3, respectively.[7] Positive predictive value of screening test and the further assessment was 11 and 77%, respectively.[7,8] The general assessment using early indicators reveals that diagnostic service conforms to the European standards on an acceptable or even desirable level.[9] Because these operational objectives are accomplished, the program has a chance to replicate a significant reduction in breast cancer mortality achieved in randomized trials. On the other hand, the potential benefits of screening need to be carefully balanced against the financial burden for the national health care system.[10] However, except for our preliminary report from the first year of screening, no other complex analysis of breast cancer detection costs has been done yet.[11]

The aim of this study was to evaluate the cost-effectiveness of population-based mammographic screening in the region of Lower Silesia.

Material and methods

Population-based breast cancer screening in Poland has been fully introduced on January 1, 2007 (after a pilot phase in 2006) by the Polish National Health Fund. This nation-wide program targets women aged 50–69 that are not undergoing treatment or being followed-up due to breast cancer. Personal invitation letters are issued centrally by the National Health Fund based on its population register. Two-view screen-film mammography, craniocaudal and oblique, is used as a standard screening test. Routine round length of the program is 2 years. All women with radiological findings categorized as BIRADS 3–5 are recalled for assessment. The second level diagnostic tools are breast clinical examination, additional imaging, and invasive investigations if needed. Following further assessment women are referred to the mammography at the routine round length of the program (benign lesions), to the short-term follow-up after 6 months (lesions of uncertain potential of malignancy), or to the treatment if breast cancer had been detected on biopsy (core-needle or vacuum-assisted).

Data was collected in a prospective manner using SIMP computer system (the official electronic system for monitoring of prophylaxis programs), as well as the databases of the regional branch of National Health Fund and the Lower Silesian Cancer Registry. The amount of expenses during the each analyzed year was obtained from the Regional Coordinating Center for Screening Programs. The number of screen-detected cancers comprises both ductal carcinomas in situ and invasive breast cancers. Costs of invasive investigations are included into the costs of the further assessment with the exception of vacuum-assisted biopsy that is reimbursed separately. Costs were measured, converted into US dollars (USD), and expressed in 2015 USD using the comparison of purchasing power of money calculated with the Consumer Price Index (CPI). Calculations were done using the database of Polish National Bank (www.nbp.pl) to determine exchange rates and the Measuring Worth service to measure the worth of amounts in USD over the time (www.measuringworth.com).

Results

The expense for screening mammograms in the initial round (2007–2008), first (2009–2010), second (2011–2012) and third (2013–2014) subsequent rounds was 4 051 666, 5 453 418, 5 767 905, and 5 244 519 USD, while the cost of invitation letters was 135 667, 144 320, 188 130, and 144 886, respectively. Expenses connected to further assessment were 207 167, 208 739, 183 991, and 163 615 USD. Operating costs related to the activity of the Regional Coordinating Center for Screening Programs included screening promotion, advertising campaigns, courses for health professionals, as well as the maintenance of information phone-line and website, data collection and analysis. These costs amounted to 337 883, 237 032, 344 808, and 347 773 USD. To sum up, the total expense for the screening program was 4 732 383, 6 043 509, 6 484 834, and 5 900 793 USD, whereas the number of cancer detect-

Table 1. Detailed cost of breast cancer screening in the region of Lower Silesia in the years 2007–2014 expressed in 2015 USD

Expenses	2007	2008	2009	2010	2011	2012	2013	2014
Screening mammograms	1 851 666	2 200 000	2 566 667	2 886 751	3 007 820	2 760 085	2 701 437	2 543 082
Recall and further assessment	82 500	124 667	110 000	98 739	99 779	84 212	88 671	74 944
Invitation letters	99 000	36 667	38 500	105 820	93 185	94 945	90 491	54 395
Regional coordinating center	156 383	181 500	119 167	117 865	183 395	161 413	192 465	155 308
Total expense	2 189 549	2 542 834	2 834 334	3 209 175	3 384 179	3 100 655	3 073 064	2 827 729
Cancers detected[1]	543	506	463	524	666	646	537	533
Expense per cancer detected	4032	5025	6122	6124	5081	4800	5723	5305

[1] invasive cancers and ductal carcinomas in situ.

ed was 1 049, 987, 1 312, and 1 070. The cost-effectiveness ratio obtained in the program for each year was 4 511, 6 123, 4 943, and 5 515 USD per cancer found. During the 8 years of program, the expense for screening mammograms and further assessment was 20 517 508 and 763 512, respectively, while the cost of invitation letters and the operating cost of the Regional Coordinating Center was 613 003 and 1 267 496, respectively. The total expense for screening program and the number of detected cancers was 23 161 519 and 4 418, respectively. The average cost of breast cancer detection in screening program in the region of Lower Silesia in years 2007–2014 was 5 243 USD. The year-by-year analysis is presented in details in Table 1.

Discussion

To compare the costs and effects among different screening programs is difficult. Cost-effectiveness of mammographic screening generally favors the organized and centralized programs, mainly because of better organization, attendance rate, extended invitation scheme covering a large part of the eligible population and comprehensive quality assurance procedures.[10] Published cost-effectiveness ratios may differ tremendously, but are often the result of different methods of calculation, time periods considered, including or excluding downstream cost. The effects of screening depend on many factors, such as the epidemiology of disease, the health care system, the quality of program, and the costs of health care.[12] Estimation using a computer model showed that these marked differences make it impossible to set up one uniform policy for all the countries.[10,12] When we evaluate the cumulative expense for mammographic screening with regard to the number of cancers found, our program looks favorable. If we assess the similar period at the start of program, the reported cost of cancer detection expressed in 2014 USD varied from 11 385 (Italy, 1991–1992) to 12 925 (Spain, 1995–1996).[13,14] Our screening seems to be much more cost-effective but the limitations of that comparison should be kept in mind. This difference is hard to explain. It might have been influenced by many factors, since the number of cancers detected is the product of screening sensitivity, incidence of breast cancer in the eligible population, and the percentage of women screened.[15] Moreover, lower reimbursement rates and salary levels in the Polish health care system compared to those in Western Europe are among many other possible explanations.

Burnside et al. used a special economic model to simulate mammography and estimate the aggregate cost and quality of screening in the US. The model generates the total USD (2001) spent on screening for the base case assumptions. Assuming approx. 65% attendance rate, a 4–7.8% recall rate, and a 30–35% cancer to biopsy ratio, the total cost would be 4 517 billion USD while the number of biopsies performed would be 337 000.[15] Through this model, the costs and cancers detected can be evaluated using different recall and biopsy characteristics. This model shows that approaching the screening goal of maximum participation (100%) would produce extremely different cost characteristics depending on the practice style parameters. The low recall rate (2.5–3.5%) and high cancer to biopsy ratio (47%) style saves over 697 million USD and over 137 000 biopsies as compared with the base case. On the other hand, the high recall rate (11%) and low cancer to biopsy ratio (< 11%) style would cost almost 3 198 billion USD more than the base case and would generate 1 156 000 more biopsies. An analysis of the number of women who can be screened and evaluated for a fixed expenditure also reflects the financial impact of distinct practice characteristics. For example, if 4 billion USD is available for breast cancer screening, the program screens (using the base case values) 67.3% of the population; 87 968 cancers will be diagnosed, and 295 000 women will undergo biopsy. This program, which uses a low recall rate and high cancer to biopsy ratio screens 75.5% of the population; 98 541 cancers will be detected, and 244 000 women will undergo biopsy. The benefit is an additional 10 000 breast cancers diagnosed early by mammography with a reduction in over 50 000 biopsies. In contrast, a program performed with high recall rate and a low cancer to biopsy ratio would screen only the 50.1% of the eligible population; 713 000 women will

undergo biopsy, yet only 65 911 cancers will be detected. For the same health care USD, an excess of 418 000 biopsies are carried out as compared with the base case. Despite this aggressive style, more than 22 057 cancers go undetected by mammography because of the low participation rate.[15]

Theoretic models suggest that the marginal cost per year of life saved by screening can be reduced up to 23% with substitution of minimal-invasive biopsy for open surgery.[16] Liberman et al. estimated that stereotactic biopsy could decrease cost of diagnosis by 50% with annual national reduction in cost approaching 200 billion USD.[17] Rubin et al. compared the cost-effectiveness of distinct methods of invasive investigation in their analysis of consecutive biopsy procedures for non-palpable mammographic abnormalities performed from 1984 (needle-localized surgery) to 1998 (vacuum-assisted core biopsy). Increased use of minimal-invasive techniques, instead of open biopsies, resulted in a significant decrease in the total expense for cancer detection from 19 503 to 14 490 USD (1998).[18] If expressed in 2014 USD, the cost of breast cancer diagnosis is reduced from 28 380 to 21 010 USD, which gives an important 7 370 USD saving per each cancer case.

The studies mentioned above clearly indicate that some performance indicators can reflect the cost-effectiveness of the screening program. Taking this under consideration, our service seems to work well. The recall rate is acceptable (in the base case range), minimal-invasive biopsy rate (core-needle and vacuum-assisted) is on a desirable level (95%), while the cancer to biopsy ratio is very high (72%).[7,8] The main disadvantage of our analysis is the fact that since vacuum-assisted biopsy is reimbursed separately, its cost is not included into a total expense for screening program. For the attendees' convenience, over 90% of screening mammograms are completed in many small services outside our hospital in strict accordance to high quality standards. However, a vast majority of the vacuum-assisted procedures in the region of Lower Silesia and almost all of these biopsies for screen-detected abnormalities are performed in our institution. Hence, some conclusions can be drawn. The reimbursement rate for vacuum-assisted procedure in Poland between 2007 and 2009 was approximately 1 000 USD. This type of biopsy offers considerable advantages, but because of the limited budget it was generally reserved for microcalcifications (under stereotactic guidance) and for very small mass lesions (ultrasound-guided). In 2007 the number of cancers found in the screening program was 543. As we reported elsewhere, in the same year a minimal-invasive biopsy rate was 95%, while a benign to malignant ratio was 1 : 2.55, which gives a cancer to biopsy ratio as high as 72%.[19] To confirm a malignant histology of screen-detected lesions, a total number of core-needle and vacuum-assisted biopsies performed in that year was 254 and 462, respectively. If we include the additional cost of vacuum-assisted procedures (462 000 USD) into the total expense for screening program the cost of cancer detection will rise up to 4 517 USD, which still remains low.

In conclusion, our findings indicate that because of low cancer detection cost a population-based mammographic screening conforming the European quality standards is cost-effective for emerging economies. It should be an important part of the public policy even in countries with strongly limited health budget.

References

1. Maciejczyk A. New prognostic factors in breast cancer. *Adv Clin Exp Med*. 2013;22:5–15.
2. Wojnar A, Puła B, Podhorska-Okołów M, Dzięgiel P. Discrepancies between HER2 assessment form core needle biopsies and surgical specimens of invasive ductal breast carcinoma. *Adv Clin Exp Med*. 2013;22:27–31.
3. Zelle SG, Baltussen RM. Economic analyses of breast cancer control in low- and middle-income countries: A systematic review. *Syst Rev*. 2013;2:20.
4. Coleman MP, Forman D, Bryant H, et al. Cancer survival in Australia, Canada, Denmark, Norway, Sweden, and the UK, 1995–2007 (the International Cancer Benchmarking Partnership): An analysis of population-based cancer registry data. *Lancet*. 2011;377:127–138.
5. DeSantis C, Siegel R, Bandi P, Jemal A. Breast cancer statistics 2011. *CA Cancer J Clin*. 2011;61:409–418.
6. Levi F, Lucchini F, Negri E, La Vecchia C. Continuing declines in cancer mortality in the European Union. *Ann Oncol*. 2008;18:593–595.
7. Szynglarewicz B, Matkowski R, Kasprzak P, et al. The effectiveness of population-based breast cancer screening programme. *Pol Merkur Lekarski*. 2009;26:117–120.
8. Matkowski R, Szynglarewicz B. First report of introducing population-based breast cancer screening in Poland: Experience of the 3-million population region of Lower Silesia. *Cancer Epidemiol*. 2011;35:e111–115.
9. Perry N, Broeders M, de Wolf C, Tornberg S, Holand R, von Karsa L. European guidelines for quality assurance in breast cancer screening and diagnosis. Fourth edition – summary document. *Ann Oncol*. 2008;19:614–622.
10. De Koning HJ. Breast cancer screening: Cost-effective in practice? *Eur J Radiol*. 2000;33: 32–37.
11. Szynglarewicz B, Matkowski R. Low cost of cancer detection in the first year of mammographic screening in Poland. *Breast*. 2011;20:585.
12. van Ineveld BM, van Oortmarssen GJ, de Koning HJ, Boer R, van der Maas PJ. How cost-effective is breast cancer screening in different EC countries? *Eur J Cancer*. 1993;29A:1663–1668.
13. Spagnolo G, Zappa M, Paci E, Giorgi D, Rosselli del Turco M. Evaluation of the costs of mammographic screening program in the city of Florence. *Epidemiol Prev*. 1995;19:330–337.
14. Plans P, Casademont L, Salleras L. Cost-effectiveness of breast cancer screening in Spain. *Int J Technol Assess Health Care*. 1996;12: 146–150.
15. Burnside E, Belkora J, Esserman L. The impact of alternative practices on the cost and quality of mammographic screening in the United States. *Clin Breast Cancer*. 2001;2:145–152.
16. Lindfors KK, Rosenquist CJ. Needle core biopsy guided with mammography: A study of cost-effectiveness. *Radiology*. 1994;190:217–222.
17. Liberman L, Fahs MC, Dershaw DD, et al. Impact of stereotaxic core biopsy on cost of diagnosis. *Radiology*. 1995;19:633–637.
18. Rubin E, Mennemeyer ST, Desmond RA, et al. Reducing the cost of diagnosis of breast carcinoma: impact of ultrasound and imaging-guided biopsies on a clinical breast practice. *Cancer*. 2001;91:324–332.
19. Szynglarewicz B, Matkowski R, Kasprzak P, et al. Diagnostic service effectiveness during the first year of breast cancer screening in the region of Lower Silesia. *Adv Clin Exp Med*. 2008;17:661–666.

Frequency of infection with *Helicobacter pylori* isolates of different antimicrobial profiles in children and adolescents: A preliminary study

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Abstract

Background. *Helicobacter pylori* (*H. pylori*) infection can occur as a mixed infection caused by several strains of *H. pylori*.

Objectives. The aim of the study was to determine the frequency of colonization of the gastric mucosa by strains of *H. pylori* with different susceptibility to antimicrobial agents.

Material and methods. The study was carried out on gastric biopsies taken from 54 previously untreated Polish children and adolescents. Of the 15 positive cultures, from each primary medium, 6 single *H. pylori* colonies were isolated, making a total of 90 isolates, and the susceptibility to metronidazole (MZ), amoxicillin (AC) and clarithromycin (CH) was determined by E-test method. The presence of the *cagA* gene and *vacA* alleles (s1, s2, m1, m2) was determined by PCR.

Results. Positive culture for *H. pylori* was noted in 15/54 (27.7%) of patients. All *H. pylori* isolates were susceptible to AC, 27.8% were resistant to MZ and 38.9% to CH. The results showed 7/15 (46.7%) of children were infected with *H. pylori* strains with antibiotic heteroresistance, resistant to CH (5/15, 33.3%) and to MZ (2/15, 13.3%). The *cagA* + *vacA* s1/m2 combination was predominant genotype among detected *H. pylori* strains. The isolates possessing different antimicrobial susceptibility profiles in the same patient were identified.

Conclusions. Microbiological analyses confirmed the presence of isolates possessing different antimicrobial susceptibility profiles in 47% of examined children with *H. pylori* infection. Different antimicrobial susceptibility profiles of *H. pylori* isolates detected in the same patient may influence the success of eradication therapy.

Key words: mixed infections, resistance, genotypes

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Helicobacter pylori (*H. pylori*) infections affect more than half of the global population. Most infected people live in developing countries, where the infection rate can be as high as 90%.[1] In Poland, 58% are infected and the incidence of infection increases with age, whereas approximately 32% of children under 18 years of age are infected.[2] In most cases, the infection is asymptomatic, but it may have a chronic course. Early infection carries a greater risk of developing diseases of the upper gastrointestinal tract, such as peptic ulcers and gastric cancer in the future.[1,3] Resistance to antibiotics and chemotherapeutics are the primary causes of eradication failure and the high costs of treatment. Furthermore, it is believed that in some individuals a mixed infection with several strains of *H. pylori* with varying antibiotic susceptibility can be present. These strains may be isolated from biopsies taken from different locations in the stomach or the mixed infection can be identified by isolating several strains from one biopsy. However, data on this topic is scarce.[4] Drug resistance is a worldwide problem. There are some differences in the primary resistance to clarithromycin (CH) and metronidazole (MZ) in different countries.[5,6] In Poland, the primary and secondary resistance of *H. pylori* strains to CH is up to 22 and 54%, whereas primary and secondary resistance to MZ is 41 and 68%, respectively.[2] *H. pylori* strains are characterized by genetic diversity. It is known that the differences in virulence of these organisms depend on various alleles of the *vacA* gene and the presence of pathogenicity island CagA containing *cagA* gene. The presence of the *cagA* gene and specific variants of the *vacA* gene are related to the level of cytotoxic activity of the pathogen as well as to the clinical consequences of the infection. Determination of certain genotypes can be the basis of the analysis of the diversity of strains in infected individuals.[7–9] The aim of the study was to determine the frequency of colonization of the gastric mucosa by strains of *H. pylori* with different susceptibility to antimicrobial agents within individual patients.

Material and methods

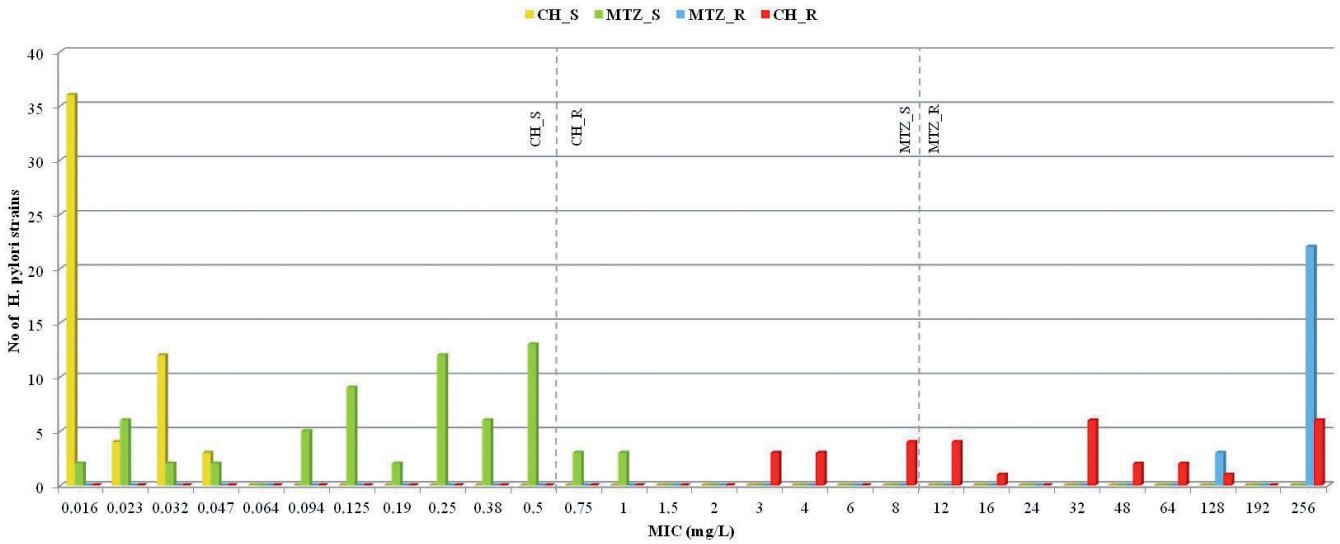
The study was conducted on gastric biopsy specimens taken from 54 consecutive children aged 3–18 years (median 15 years) who were diagnosed and treated in the 2nd Department of Pediatrics, Gastroenterology and Nutrition at the Wroclaw Medical University, Poland during January, February, and March 2014. Patients had not been previously diagnosed and treated for *H. pylori* infection. Patients who had previously had *H. pylori* infection or received antibiotics within the last 2 months were excluded from the study. A total of 4 gastric biopsy specimens were taken during endoscopy of the upper gastrointestinal tract from each child: 2 biopsies from the antrum and 2 from the corpus for histopathology and microbiology examinations. The biopsy specimens were evaluated histologically according to the Updated Sydney System.[10] Biopsies collected for microbiological examination were placed immediately after collection in sterile saline (0.15 M NaCl) and processed within 2 h at the Department of Microbiology, Wroclaw Medical University. The isolation and identification of strains were performed as described previously.[11] After obtaining a positive culture, 6 single colonies of *H. pylori* were isolated from each medium (with special attention to differences in the size of colonies) and transferred to new plates with Columbia agar supplemented with 7% of hemolyzed horse blood which were incubated for 3–4 days in microaerophilic conditions. Of the 15 positive cultures, 6 single *H. pylori* colonies were isolated from each primary medium, making a total of 90 isolates. Susceptibility of strains to the antibiotics amoxicillin (AC), clarithromycin (CH), and metronidazole (MZ) was determined by gradient diffusion (E-test, BioMerieux) on Mueller-Hinton agar (Oxoid) supplemented with 10% horse blood under microaerophilic conditions.[11] The strains were considered resistant to AC, CH and MZ when the minimum inhibitory concentration (MIC) values were > 0.12, > 0.5 and > 8 mg/mL, respectively.[11] DNA isolation and determination of the *cagA* gene and *vacA* alleles in isolated strains were performed as described elsewhere.[12] Statistical analysis was performed by χ^2 test with or without Yates' correction. A p-value < 0.05 was considered significant for all tests. Statistical analyses were done by using STATISTICA v. 10.0 software (StatSoft).

The study was undertaken according to the Declaration of Helsinki. Informed written consent was obtained from all parents and from patients older than 16 years of age.

Results

On the basis of endoscopy and histopathology findings the 54 examined children were diagnosed with gastric ulcer (n = 1), duodenal ulcer (n = 2), ulcers of the stomach and duodenum (n = 1), chronic gastritis (n = 34), and other gastrointestinal disorders (n = 16) including gastroesophageal reflux disease, celiac disease, lactose intolerance, impaired intestinal absorption with low body weight, irritable bowel syndrome, dyspepsia, Crohn's disease and ulcerative colitis. *H. pylori* infection was detected in 16/54 (29.6%) of children and a positive culture for *H. pylori* was noted in 15/54 (27.7%) of patients. All tested strains were sensitive to AC (MIC ≤ 0.016 mg/L). The percentage of *H. pylori* strains resistant to MZ was 27.8% and to CH 38.9%. One patient was infected with a strain resistant to both MZ and CH. Strains resistant to CH showed varying degrees of resistance (MIC, 0.5–256 mg/L) and strains resistant to MZ showed a high degree of resistance (MIC, 8–256 mg/L) (Fig. 1). In 8/15 (53.3%) of patients, *H. pylori* strains possessed the same antimicrobial susceptibility profiles. *H. pylori* strains with heteroresistance to MZ and CH were detected in 7/15 (46.7%) of children. Infection

Fig. 1. MIC values for *H. pylori* isolates obtained from gastric biopsies



MZ – metronidazole, CH – clarithromycin.

with *H. pylori* strains with heteroresistance to CH and to MZ was demonstrated in 5/15 (33.3%) and 2/15 (13.3%) of patients, respectively (Table 1). Three patients (no. of patients: 6, 8, 14) were infected with strains resistant and sensitive to CH, whereas, in 2 children, *H. pylori* strains resistant to CH, but with significantly different MIC values (32 and 256) were isolated (patient 1 and 3) (Table 2). In 2 children, isolates with different resistance to CH were identified from corpus biopsies, while in isolates from the antrum differences in susceptibility to antibiotics were not observed. Genetic analyses, determining the presence of the *cagA* gene and *vacA* alleles (s1, s2, m1, m2) were done in 22 selected *H. pylori* isolates, of which 14 strains with heteroresistance profiles were isolated from children with mixed infection, and 8 strains were isolated from patients with infections caused by a single strain. The most common infection was caused by strains with the *cagA*+ *vacA* s1/m2 genotype (36.4%). Correlation between susceptibility profile and bacterial genotypes is present in Table 3. Strains resistant to MZ were *cagA*– (66.7%) and *cagA*+ (33.3%), and the difference was statistically significant (p = 0.0003). In examined children with culture positive for *H. pylori*, the strains possessing the s1 allele and *cag*A gene were predominant. The *vacA* s2/m2 genotype was more common in children with chronic gastritis.

Discussion

The analysis of data from 10 countries in different continents showed a large variation of resistance of *H. pylori* to CH in children (13.9–84.9%).[13] The high percentage of resistance is probably due to the increased use of CH in the treatment of respiratory infections in children.[13] Re-

Table 1. Analysis of antimicrobial susceptibility profile of *H. pylori* strains in examined patients

MZ + CH combination	Patients with positive culture for *H. pylori* (n = 15)			Number of patients with negative culture for *H. pylori*	Number of examined children
	number of patients with strains of the same antimicrobial susceptibility profile	number of patients with strains of different antimicrobial susceptibility profile			
	MZ + CH	MZ	CH		
MZ-S, CH-S	4/15 (26.7%)	–	–		
MZ-R, CH-S	2/15 (13.3%)	–	–		
MZ-S, CH-R	2/15 (13.3%)	–	–		
MZ-R+S, CH-S	–	2/15 (13.3%)	–		
MZ-S, CH-R+S	–	–	3/15 (20%)		
MZ-S, CH-R+R	–	–	1/15 (6.7%)		
MZ-R, CH-R+R	–	–	1/15 (6.7%)		
Total	8/15 (53.3%)	7/15 (46.7%)		39	54

MZ – metronidazole; CH – clarithromycin; No. – number; S – sensitive; R – resistant; R+R – heteresistance of isolates isolated from the same patient; R+S – isolates resistant and sensitive isolated from the same patient.

Table 2. Characteristics of *H. pylori* isolates with different sensitivity to clarithromycin and metronidazole

Number of patient	Source of biopsy	Number of isolate	MZ		CH	
			MIC [mg/L]	S/R	MIC [mg/L]	S/R
1	antrum	1, 2	1	S	256	R
		3	0.25		256	
		4, 5	0.5		32	R
		6	1		32	
2	antrum	1, 2, 3	256	R	0.016	S
		4, 5	0.25	S	0.016	
		6	0.19		0.016	
3	antrum	1, 2	256	R	256	R
		3	128		256	
		4, 5, 6	256		32	R
4	antrum	1, 6	256	R	0.016	S
		2	256		0.023	
		3	128		0.016	
		4	128		0.032	
		5	256		0.032	
5	antrum	2, 3	0.25	S	4	R
		1, 4, 5	0.38		3	
		6	0.19		4	
6	corpus	1	0.5	S	0.016	S
		2, 6	0.75		48	R
		3	0.5		0.016	S
		4	0.75		64	R
		5	0.5		64	
7	antrum	1, 5	0.125	S	8	R
		2, 6	0.094		8	
		3	0.094		12	
		4	0.125		12	
8	corpus	1	0.023	S	16	R
		2, 3	0.047		12	
	antrum	4, 5, 6	0.023	S	0.016	S
9	antrum	1, 2, 3	0.125	S	0.016	S
		4	0.125		0.023	
		5	0.094		0.023	
		6	0.094		0.016	
10	antrum	2	0.25	S	0.032	S
		1, 3, 4	0.5		0.016	
		5, 6	0.5		0.032	
11	antrum	1	256	R	0.023	S
		2, 3	256		0.016	
		4, 5, 6	256		0.032	
12	antrum	1, 6	0.023	S	0.016	S
		2, 3	0.016		0.016	
		4, 5	0.032		0.032	
13	antrum	1, 6	256	R	0.016	S
		2	0.125	S	0.016	
		3	0.125		0.047	
		4, 5	256	R	0.047	

Table 2. Characteristics of *H. pylori* isolates with different sensitivity to clarithromycin and metronidazole – cont.

No. of patient	Source of biopsy	No. of isolate	MZ		CH	
			MIC [mg/L]	S/R	MIC [mg/L]	S/R
14	antrum	1	0.25	S	96	R
		2, 3	0.25		0.016	S
		4, 6	0.5		96	R
		5	0.5		128	
15	antrum	1, 5	0.38	S	0.016	S
		2, 6	0.25		0.016	
		3	0.25		0.032	
		4	0.38		0.032	

MZ – metronidazole; CH – clarithromycin; S – sensitive; R – resistant; No. – number; MIC – minimal inhibitory concentration.

Table 3. Correlation between susceptibility profiles and genotypes among isolated *H. pylori* strains

Genotype		Resistant *H. pylori* strains			Sensitive *H. pylori* strains	Total
		MZ no. (%)	CH no. (%)	MZ + CH no. (%)	no. (%)	no. (%)
cagA (–)	s1/m1	–	–	–	–	–
	s1/m2	–	–	2/22 (9%)	–	2/22(9%)
	s2/m1	–	–	–	–	–
	s2/m2	2/22 (9%)	–	–	–	2/22 (9%)
	total	2/22 (9%)	–	2/22 (9%)	–	4/22(18%)
cagA (+)	s1/m1	1/22 (5%)	2/22 (9%)	–	3/22 (14%)	6/22 (27%)
	s1/m2	–	4/22 (18%)	–	4/22(18%)	8/22 (36%)
	s2/m1	–	–	–	–	–
	s2/m2	1/22(5%)	1/22(5%)	–	2/22 (9%)	4/22 (18%)
	total	2/22(9%)	7/22 (32%)	–	9/22(41%)	18/22 (82%)

MZ – metronidazole; CH – clarithromycin; no. – number.

sistance of *H. pylori* strains to MZ in children showed an even wider range (7.4–95%), which might result from differences in the frequency of treatment with MZ for various infectious diseases.[13] In European children the percentage of strains resistant to CH is higher (> 30%) than in adults (17.5%), whereas the percentage of strains resistant to MZ is lower (25.7%).[14] This study showed a high prevalence of strains resistant to both MZ and CH. The resistance to CH was higher (38.9%) than the previously reported resistance to this drug observed in children in Poland (9–29%), and the resistance to MZ (27.8%) was located in the middle of the range (16–43%).[2] *H. pylori* strains resistant to CH showed a wide range of MIC; in the strains resistant to MZ, the MIC values were very high (192–256 mg/L). In our study, all strains of *H. pylori* were susceptible to amoxicillin. These results are similar to other reports.[2,5,13] Primary resistance to amoxicillin is very rare (0–2%); however, in some countries, strains resistant to AC have been detected.[2,5,13]

Gastric mucosa can be colonized with single or multiple strains of *H. pylori*. Furthermore, this colonization may lead to the presence of many sub-populations of *H. pylori*, as the result of genetic mutation or recombination.[15] Genetic exchange of genes between different strains may contribute to the emergence of more virulent strains. It is believed that *H. pylori* undergoes a recombination with heterologous strains much more frequently than is the case with other bacterial species.[16] In addition, during exposure to the antibiotics used to treat other infections, multiresistant phenotypes of *H. pylori* may occur, which is probably associated with the instability of the genomic DNA of the pathogen as well as with the acquisition of another strain.[17] The presence of *H. pylori* infections with mixed strains confirmed with genetic analyses varies from 0 to 85% in different reports.[18] Studies in European and Western countries have shown that the majority of *H. pylori* strains isolated from different parts of the stomach possessed homogeneous DNA profiles. In contrast,

the populations of China and Mexico are often infected with mixed strains, genetically heterogeneous, which is associated with a high incidence of infection in the populations.[4,16,17] In our study, infection with *H. pylori* strains with different sensitivity to the MZ and CH was identified in 7 out of 15 children (46.7%). It can be assumed that such high levels might be the result of a high incidence of *H. pylori* infection in the Polish population. Our results also showed that clarithromycin-heteroresistance was more frequent in the analyzed strain than the resistance to metronidazole. The common use of clarithromycin in treating respiratory tract infections in Polish children may explain these findings. However, to our knowledge, this is the first study on *H. pylori* infection with antibiotic-heteroresistant strains in a Polish population, and more research on this subject is needed in Poland.

The *vacA* and *cagA* genes are used as markers of the genomic diversity of *H. pylori* strains.[8,9] In East Asia, almost all strains of *H. pylori* are *cagA*+. In Europe and Africa approximately 60–80% of the strains are *cagA*+.[19] In this study, the presence of the *cagA* gene was found in 81.8% of the strains. This result is significantly higher than that observed in other studies in Poland.[20] Two children infected with heteroresistant *H. pylori* strains showed differences in the presence of the *cagA* gene. These strains differed in the resistance to MZ; those sensitive to MZ were *cagA*+, whereas the strains resistant to MZ were *cagA*–, suggesting an infection in a single patient with different strains. Among the *H. pylori* isolates with heteroresistance isolated from the same patient, the presence of different genotypes, related to the presence of the *cagA* gene, was detected. It appears that isolates with varying resistance and with different genotypes came from a mixed *H. pylori* infection. However, in the remaining 5 children, the isolates with a different resistance and with the same genotype probably came from a single strain infection. It may be that in these patients, the initially susceptible strains acquired resistance through mutation and genetic recombination, and then later, 2 subpopulations existed together in the gastric mucosa. These results require further analysis and characteristics of the genetic profile of tested strains to confirm or exclude infection with mixed strains of *H. pylori*.

Conclusion

In conclusion, in 47% of the examined children infected with *H. pylori*, microbiological analyses confirmed the presence of isolates possessing different antimicrobial susceptibility profiles, and the infection was more commonly caused by strains resistant to CH. Among the isolates with different resistance to CH and MZ isolated from the same patient, different genotypes were detected, which may suggest a mixed infection. Different antimicrobial susceptibility profiles of *H. pylori* isolates detected in the same patient may influence the success of eradication therapy.

References

1. Khalifa MM, Sharaf RR, Aziz RK. *Helicobacter pylori*: A poor man's gut pathogen? *Gut Pathog*. 2010;2:2.
2. Laszewicz W, Iwańczak F, Iwańczak B, Task Force of the Polish Society of Gastroenterology. Seroprevalence of *Helicobacter pylori* infection in Polish children and adults depending on socioeconomic status and living conditions. *Adv Med Sci*. 2014;59:147–150.
3. Salama NR, Hartung ML, Müller A. Life in the human stomach: Persistence strategies of the bacterial pathogen *Helicobacter pylori*. *Nat Rev Microbiol*. 2013;11:385–399.
4. Raymond J, Nguyen B, Bergeret M, Dupont C, Kalach N. Heterogeneous susceptibility to metronidazole and clarithromycin of *Helicobacter pylori* isolates from a single biopsy in adults is confirmed in children. *Int J Antimicrob Agents*. 2005;26:272–278.
5. De Francesco V, Giorgio F, Hassan C, et al. Worldwide *H. pylori* antibiotic resistance: A systematic review. *J Gastrointestin Liver Dis*. 2010;19:409–414.
6. Ghotaslou R, Leylabadlo HE, Asl YM. Prevalence of antibiotic resistance in *Helicobacter pylori*: A recent literature review. *World J Methodol*. 2015;5:164–174.
7. Iwanczak B, Laszewicz W, Iwanczak F, et al. Genotypic and clinical differences of seropositive *Helicobacter pylori* children and adults in the Polish population. *J Physiol Pharmacol*. 2014;65:801–807.
8. Matos JI, de Sousa HA, Marcos-Pinto R, Dinis-Ribeiro M. *Helicobacter pylori cagA* and *vacA* genotypes and gastric phenotype: A meta-analysis. *Eur J Gastroenterol Hepatol*. 2013;25:1431–1441.
9. Sgouras DN, Trang TT, Yamaoka Y. Pathogenesis of *Helicobacter pylori* Infection. *Helicobacter*. 2015;20(Suppl 1):8–16.
10. Dixon MF, Genta RM, Yardley JH, et al. Classification and grading of gastritis. The updated Sydney System. *Am J Surg Pathol*. 1996;20:1161–1181.
11. Gościniak G, Biernat M, Grabińska J, Bińkowska A, Poniewierka E, Iwańczak B. The antimicrobial susceptibility of *Helicobacter pylori* strains isolated from children and adults with primary infection in the Lower Silesia Region, Poland. *Pol J Microbiol*. 2014;63:57–61.
12. Biernat MM, Gościniak G, Iwańczak B. Prevalence of *Helicobacter pylori cagA*, *vacA*, *iceA*, *babA2* genotypes in Polish children and adolescents with gastroduodenal disease. *Postepy Hig Med Dosw*. 2014;68:1015–1021.
13. Megraud F, Coenen S, Versporten A, et al. *Helicobacter pylori* resistance to antibiotics in Europe and its relationship to antibiotic consumption. *Gut*. 2013;62:34–42.
14. Seo JH, Woo HO, Youn HS, Rhee KH. Antibiotics resistance of *Helicobacter pylori* and treatment modalities in children with *H. pylori* infection. *Korean J Pediatr*. 2014;57:67–71.
15. Secka O, Antonio M, Berg DE, et al. Mixed infection with *cagA* positive and *cagA* negative strains of *Helicobacter pylori* lowers disease burden in The Gambia. *PLoS One*. 2011;6(11):e27954.
16. Patra R, Chattopadhyay S, De R, et al. Multiple infection and microdiversity among *Helicobacter pylori* isolates in a single host in India. *PLoS One*. 2012;7(8):e43370.
17. Kao CY, Lee AY, Huang AH, et al. Heteroresistance of *Helicobacter pylori* from the same patient prior to antibiotic treatment. *Infect Genet Evol*. 2014;23:196–202.
18. Farzi N, Malekian T, Alebouyeh M, Vaziri F, Zali MR. Genotype diversity and quasispecies development of *Helicobacter pylori* in a single host. *Jpn J Infect Dis*. 2015;68:176–180.
19. Suzuki R, Shiota S, Yamaoka Y. Molecular epidemiology, population genetics, and pathogenic role of *Helicobacter pylori*. *Infect Genet Evol*. 2012;12:203–213.
20. Zalewska-Ziob M, Wiczkowski A, Strzelczyk JK, et al. The prevalence of *Helicobacter pylori vacA* alleles in patients with chronic gastritis. *Adv Clin Exp Med*. 2007;16:29–33.

Clinical risk factors for loss of stent primary patency in patients with chronic legs ischemia

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Abstract

Background. The outcome of endovascular therapy can be influenced by a number of factors, either demographic, biochemical, angiographic or procedural. Knowledge about these factors may help in the individualization of therapeutic methods, surveillance intensity, and should, ultimately, improve intervention efficacy.

Objectives. The aim of this study was to estimate the effect of clinical and biochemical factors on the late outcome of lower limb artery stenting.

Material and methods. The medical documentation of 91 patients with at least a 1 year follow-up after the stenting of a lower limb artery was retrospectively evaluated. Uni- and multivariate analyses were performed.

Results. Primary patency within an approximately 1.5-year follow-up amounted to 68.2%. The probability of freedom from target lesion revascularization was significantly greater in patients with dyslipidemia. According to the Cox proportional-hazards analysis, the risk of target extremity revascularization was significantly affected by the following (hazard ratio [HR], 95% confidence interval): Age (0.93, 0.88–0.99); dyslipidemia at inclusion (0.046, 0.01–0.23); LDL blood concentration (1.02; 1.01–1.04); hematocrit (1.2, 1.02–1.42); mean platelet volume (0.66, 0.44–0.99); INR (1.58, 1.13–2.21); and aPTT (1.18, 1.07–1.3).

Conclusions. Endovascular treatment with stenting in patients with atherosclerotic peripheral arterial disease is effective, but the risk of primary patency loss was affected by the presence of dyslipidemia, age, and blood coagulation parameters. The effect of dyslipidemia on stent failure occurrence should be evaluated in further studies.

Key words: risk factors, dyslipidemia, peripheral artery disease, in-stent restenosis, blood coagulation

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The endovascular treatment of peripheral artery disease (PAD) is still progressing and is associated with many complications that reduce its later benefits, such as in-stent thrombosis, stent fracture, in-stent restenosis (ISR) and stent infection.[1,2] A common factor affecting the outcome of endovascular therapy is atherosclerosis progression, which requires consecutive limb revascularization outside the primary treated site. One year after an endovascular procedure, the primary patency of a stent implanted into an iliac artery amounts to 81–94%, and 60–82% for stents implanted into a femoropopliteal segment of the vascular bed, depending on the indications for the procedure (claudication or critical limb ischemia, CLI) and lesion severity (stenosis or total occlusion).[1,2]

Advances in special equipment dedicated to endovascular therapy has helped to reduce the prevalence of the above-mentioned complications, and offer the possibility of treating stent failure. Among such techniques are the use of: a) percutaneous angioplasty using plain old balloon angioplasty (POBA), drug-eluting balloons (DEB), and cutting balloons or cryoplasty; b) different scaffold techniques with the use of bare metal stents (BMS), drug-eluting stents (DES), cytokine- or growth-factor-eluting stents, covered stents, and bare or drug-eluting bioresorbable vascular scaffolding (BVS); as well as c) debulking techniques (atherectomy, and turbo-booster and excimer lasers).[1,3–12] Few of these modern techniques are widely available, and many are also more aggressive and expensive. Therefore, they cannot be used for all patients. For this reason, the proper identification of patients with a high risk of loss of stent primary patency, which may be an effect of both atherosclerosis progression and smooth muscle (neointima) proliferation, may help in the individualization of patients' qualification for respective treatment methods and, in this way, improve percutaneous therapy outcome. Such patients might also have different surveillance patterns. However, the majority of papers focus mainly on the following predictors of the primary patency of stenting: angiographic lesion severity (stenosis or occlusion), procedural factors (indications for procedure [claudication vs critical limb ischemia], type of patency restored [subintimal or intraluminal], length of stent, and outflow state).[2,3,11–14] For this reason, we analyzed the effect of clinical and biochemical factors on the outcome of lower limb artery stenting.

Material

The medical documentation of 91 consecutive patients treated endovascularly between 2008 and 2013 with lower extremity artery stenting due to atherosclerosis was retrospectively evaluated. The inclusion criteria were as follows: a) first-time stenting of lower extremity arteries due to chronic leg ischemia; b) complete follow-up, for at least 1 year with visits at 1, 3, 6, and 12 months after the endovascular procedure, and then at least every 6 months. The exclusion criteria were: a) stent occlusion due to thrombosis or fracture, b) lack of compliance with suggestion regarding a change in lifestyle and the taking of medicines, and c) surgical revascularization during follow-up after stenting.

Percutaneous procedures in aorto-iliac level were performed in 26 (28.6%) patients. Among them 4 (15%) had grade A of lesion severity according to TASC classification, 4 (15%) grade B, 10 (38.5%) grade C, and 8 (31.5%) grade D.[2] In femoro-popliteal segment interventions were done in 65 (71.4%) individuals. Of them, 4 (6%) patients had lesions in the severity of grade A, 14 (21%) grade B, 20 (31%) grade C, and 27 (42%) grade D. Twenty five (27.5%) patients with intervention in femoro-popliteal segment needed additional angioplasty in the arteries below the knee to improve run-off. One stent was implanted in 49/91 (54%) subjects. Two stents were introduced in 10/26 (38%) patients with the lesions in aorto-iliac segment, and 32/65 (49%) individuals with femoro-popliteal intervention. Thirty percent of patients were treated due to critical limbs ischemia (Table 1).

Table 1. Clinical data of patients with and without TLR

Parameter	TLR n = 29	TLR-free n = 62	p-value
Age (years)	63.2 ± 9.5	62.9 ± 8.4	0.86
Gender (male, n%)	24 (83%)	49 (79%)	0.68
Critical limb ischemia (n, %)	12 (41.4%)	15 (24.2%)	0.078
Smoking habit (n, %)	14 (48%)	33 (53%)	0.66
Hypertension (n, %)	19 (66%)	44 (71%)	0.60
Diabetes mellitus (n, %)	10 (35%)	16 (26%)	0.40
Fasting glucose (mg/dL)	106.4 ± 35.7	106.6 ± 25.6	0.98
BMI (kg/m^2)	25.5 ± 4.8	28.5 ± 4.8	0.14
History of CAD (n, %)	13 (45%)	25 (42%)	0.78
History of stroke (n, %)	4 (14%)	5 (8%)	0.43
History of CHF (n, %)	4 (14%)	12 (20%)	0.48
LDL cholesterol (mg/dL)	116.0 ± 41.7	124.8 ± 38.9	0.40
Triglycerides (mg/dL)	118.2 ± 46.0	145.1 ± 69.6	0.12
Dyslipidemia (n, %)	16 (55%)	55 (89%)	0.0002
Leucocytes count (g/L)	9.1 ± 2.6	9.1 ± 2.5	0.93
Hemoglobin (g/L)	13.3 ± 1.8	13.5 ± 1.7	0.64
Hematocrit (%)	39.7 ± 4.9	39.9 ± 4.5	0.88
MCV (fl)	88.3 ± 5.8	89.2 ± 5.5	0.52
Platelets count (g/L)	290.5 ± 115.5	271.6 ± 112.6	0.47
MPV (fl)	9.4 ± 1.5	9.5 ± 1.3	0.67
Creatinine (mg/dL)	1.04 ± 0.23	1.1 ± 0.2	0.77
INR	1.44 ± 0.2	1.04 ± 0.2	0.08
aPTT (s)	33.2 ± 9.2	30.5 ± 5.0	0.14

TLR – target lesion revascularization; BMI – body mass index; CAD – coronary artery disease; CHF – congestive heart failure; LDL – low-density lipoprotein; MCV – mean corpuscular volume; MPV – mean platelet volume; INR – international normalized ratio; aPTT – activated partial thromboplastin time. Some data was also presented earlier.[13]

Methods

According to the reference data, the following parameters were taken into consideration for analysis: age, gender, symptoms, smoking habits, hypertension, dyslipidemia (defined as low-density lipoprotein [LDL] cholesterol blood concentration at admission for a first endovascular procedure ≥ 100 mg/dL, and/or triglyceride blood concentration on admission for a first endovascular procedure ≥ 150 mg/dL in spite of hypolipemic therapy with statins), diabetes mellitus, history of coronary artery disease (CAD), percutaneous coronary intervention (PCI), coronary artery bypass graft (CABG), past stroke, history of congestive heart failure (CHF), chronic kidney disease with blood creatinine level of > 2 mg/dL, weight, height, body mass index (BMI), bilateral ankle-brachial index (ABI), claudication distance, and biochemical parameters (LDL cholesterol, triglycerides, leucocyte count, hemoglobin concentration, hematocrit, mean corpuscular volume, platelet count, mean platelet volume [MPV], creatinine, activated partial thromboplastin time [aPTT], prothrombin time expressed as an international normalized ratio [INR], fasting blood glucose, and blood group).[15] The kind of pharmacotherapy recommended was also analyzed (aspirin, clopidogrel, angiotensin-converting enzyme inhibitor [ACEI], angiotensin receptor blocker [ARB], beta-adrenergic receptor blocker, calcium channel antagonist, statin, type of statin, ezetimibe, fenofibrate, enoxaparin, warfarin, and acenocoumarol). All subjects were treated with aspirin, statin, ACEI, and beta-blockers.

The patients were followed up 1, 3, 6 and 12 months after the procedure and then every 6 months. On each control visit, symptom severity and the result of color-coded duplex ultrasound examination were evaluated. The need for revascularization was considered when resting leg pain, or claudication limiting usual daily physical activity was accompanied by a reduction of vessel diameter by 70% or more (peak systolic velocity index > 2.4) or complete vessel occlusion appeared.

Main outcomes measured

During the period between percutaneous procedure performance and February 18, 2014 (the date of the Bioethical Committee agreement), the following main effectiveness outcomes of the analysis were taken: freedom from target lesion revascularization (TLR), target extremity revascularization (TER), and target limb amputation (TLA).[15] TLR was defined as repeat percutaneous (endovascular) revascularization for a lesion anywhere within the stent or the 5 mm border proximal or distal to the stent due to symptom recurrence and target lesion occlusion (mainly due to IRS). This was treated with angioplasty (POBA or DEB) or stent-into-stent implantation. TER was defined in the wider criteria than usual, as the revascularization of a previously stented lower limb both due to the target lesion or a new lesion at least 10 mm outside the proximal or distal border of the stent due to symptom recurrence (claudication impeding normal daily functioning of the patient or critical limb ischemia) and lesion diameter stenosis ≥ 50% (effect of atherosclerosis progression).[15] TLA was defined as minor or major amputation of a previously stented limb during the follow-up period.

The safety outcomes were also analyzed retrospectively. They were established as a major adverse event (MAE) and were as follows: in-hospital TLA as a result of endovascular procedure complication, periprocedural acute coronary syndrome (ACS), stroke, CHF exacerbation, acute kidney injury (AKI), and death. TLA was defined as a minor or major amputation of a previously stented limb during hospitalization during which an endovascular procedure was performed. Post-procedural AKI was defined as an abrupt increase in creatinine blood concentration within 7 days after an endovascular procedure.

Ethics

The study protocol was approved by the local Bioethics Committee (agreement number KB139/2014). All procedures have been conducted in compliance with the Declaration of Helsinki.

Statistical analysis

A licensed version of StatSoft, Inc. (2011), STATISTICA (data analysis software system), v. 10 was used. The normal distribution of the study variables was checked using the Shapiro-Wilk test. The results were mainly presented as the mean ± standard deviation (SD) or n, %. The statistical significance of the differences between patients needing an end-point procedure and patients not requiring such treatment was verified using the unpaired student's t-test and Fisher's exact test (Table 1). The survival analysis for the 91 subjects was carried out using Cox's F test, the log-rank test in the Kaplan-Meier method for 2 groups, and Cox proportional-hazards analysis.

Results

The mean observation time amounted to 544 ± 503 days. Within this follow-up, 29/91 (31.9%) of the subjects required TLR, and the primary stent patency amounted to 68.1%. An additional 3 patients needed an endovascular intervention with regard to lesions localized outside the previously implanted stent. For this reason, 32 (35.2%) of the patients required TER. Minor or major leg amputation was performed in 5 (5/91; 5.5%) patients. The majority of these patients required TLR (4/5, 80%; p = 0.038).

The mean period between the first endovascular procedure and TLR, TER, and leg amputation amounted to 221 ± 304 days, 264 ± 368 days, and 455 ± 521 days, respectively. The mean time between TLR and minor or major leg amputation amounted to 314 ± 484 days.

Ninety-one patients were included. Their demographic and clinical data in relation to end-point occurrence are presented in Table 1. The only significant difference between patients who required TLR (Table 1), TER or leg amputation and those who were free from these safety end-points was a lower prevalence of dyslipidemia upon admission among the former (Table 1). Diabetes mellitus, hypertension, smoking or history of cardiovascular event did not affect significantly the prevalence of TLR (Table 1, Fig. 2). When an analysis on TLR occurrence was performed separately for patients with interventions concerning aorto-iliac or femoro-popliteal segments as well as the severity of TASC grade of lesions localized in respective vascular segments, the effect of investigated clinical and biochemical risk factors on TLR prevalence was statistically not significant.[2]

Information regarding the use of the Kaplan-Meier analysis for 2 groups and the Cox regression model showed a significant negative association between the between the duration of TLR-free period and the presence of dyslipidemia at admission, which reduced the risk of TLR by about 20 times (Fig. 1). The positive correlation between TLR occurrence and aPTT and INR values on the day of the first endovascular procedure was found (Table 2). Initial BMI and MPV negatively affected the

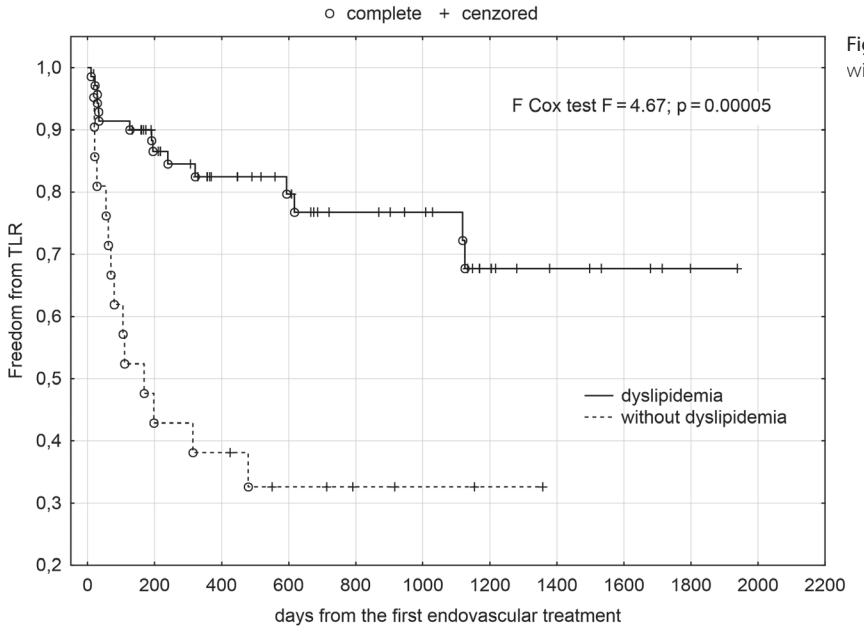

Fig. 1. Kaplan-Meier TLR-free survival curve in patients with and without dyslipidemia

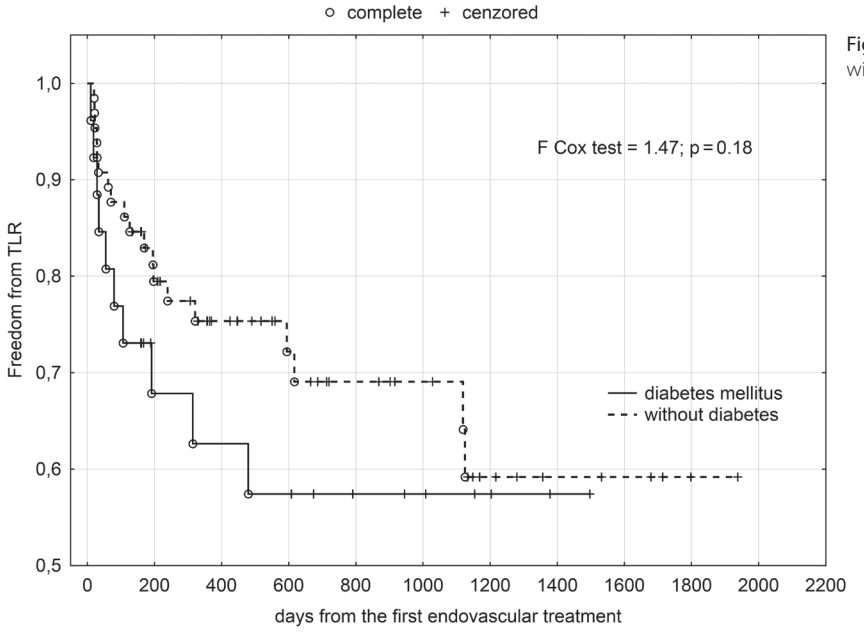

Fig. 2. Kaplan-Meier TLR-free survival curve in patients with and without diabetes mellitus

Table 2. The Cox proportional-hazards regression model for target lesion revascularization (TLR). $\chi^2 = 43.4$; p = 0.0042

Parameter	β-coefficient	β 95% CI	p-value	Hazard ratio	Hazard ratio 95% CI
Age (years)	–0.06	–0.12–0.11	0.11	0.95	0.88–1.01
Gender (female/male)	0.78	–0.71–2.26	0.31	2.17	0.49–9.56
Smoking	–0.46	–1.39–0.48	0.34	0.63	0.25–1.61
Hypertension	0.38	–0.70–1.46	0.49	1.46	0.49–4.3
Diabetes mellitus	0.81	–0.34–1.96	0.17	2.25	0.71–7.11
Dyslipidemia	–2.99	–4.65– –1.34	0.001	0.05	0.01–0.26
BMI	–0.17	–0.36–0.02	0.08	0.84	0.70–1.02
History of CAD	–0.49	–1.67–0.68	0.41	0.61	0.19–1.98
Past stroke	–0.56	–2.15–1.03	0.49	0.57	0.12–2.79
History of CHF	–0.35	–2.16–1.47	0.71	0.71	0.12–4.34
Creatinine > 2 mg/dL	1.66	–0.42–3.74	0.12	5.27	0.66–42.22
LDL cholesterol (mg/dL)	0.01	–0.01–0.03	0.21	1.01	0.99–1.03
Triglycerides (mg/dL)	–0.01	–0.02–0.01	0.60	0.99	0.98–1.01
Leucocytes count (G/L)	–0.01	–0.12–0.1	0.85	0.99	0.89–1.10
Hematocrit (%)	0.11	–0.5–0.26	0.18	1.11	0.95–1.3
MCV	–0.03	–0.13–0.08	0.64	0.97	0.87–1.08
Platelets count (G/L)	–0.01	–0.01–0.01	0.30	0.99	0.99–1.00
MPV (fL)	–0.39	–0.82–0.05	0.08	0.68	0.44–1.05
Creatinine (mg/dL)	1.65	–1.38–4.67	0.29	5.19	0.25–106.8
INR	0.92	0.42–1.42	0.001	2.52	1.52–4.16
aPTT	0.13	0.04–0.23	0.005	1.14	1.04–1.25
Fast glucose (mg/dL)	–0.01	–0.03–0.02	0.62	0.99	0.97–1.02

CI – confidence interval; BMI – body mass index; CAD – coronary artery disease; CHF – congestive heart failure; LDL – low-density lipoprotein; MCV – mean corpuscular volume; MPV – mean platelet volume; INR – international normalized ratio; aPTT – activated partial thromboplastin time.

length of freedom from TLR but only with borderline statistical significance (Table 2). Meanwhile, TER risk had a positive association with LDL blood concentration and aPTT and INR values, as well as a negative correlation with the patient's age, dyslipidemia, and MPV value at the day when endovascular procedure was performed (Table 3). No MAE occurred in any of the patients.

Discussion

For this paper, we retrospectively analyzed the medical documentation of patients undergoing endovascular treatment due to lower extremity ischemia. The standard end-points were evaluated as the outcome measure using survival analysis. We found that within an approximately 1.5-year follow-up about 30% of the patients had required TLR or TER (about 70% primary stent patency). Such endovascular therapy outcomes corroborate the results of other authors.[1,2]

The loss of primary stent patency is an effect of in-stent restenosis, related mainly to neointima proliferation, and/or atherosclerosis progression. In this study, TLR risk was significantly smaller in patients with dyslipidemia (Table 1, Table 2, Fig. 1), greater BMI (borderline), and MPV (borderline), as well as in those with shorter aPTT and INR on the day of the first endovascular procedure (Table 2). Meanwhile, TER occurrence was related to the patient's age, LDL cholesterol blood concentration, hematocrit, and MPV, aPTT and INR values (Table 3).

The results obtained (Table 1–3, Fig. 1) seem to be inconsistent with the state of art knowledge concerning pathophysiology and the management of cardiovascular disorders, due to at least 2 reasons. Firstly, we did not show a statistically significant effect of diabetes (Fig. 2), smoking, and hypertension on the risk of TLR (Table 1, 2), and secondly, we found that dyslipidemia decreased an established end-point risk. However, it is known that elevated LDL cholesterol is recognized as one of the main traditional risk factors for cardiovascular diseases, atherosclerosis development and progression, and LDL cholesterol concentration below 70 mg/dL, below 100 mg/dL, and below 115 mg/dL are proposed as its target levels during therapy depending on individual patient risk stratification.[16,17] Although risk factors for TLR in patients with lower limbs ischemia may be different than for coronary artery atherosclerosis progression, there is also some evidence that patients with continuing dyslipidemia had

Table 3. The Cox proportional-hazards regression model for target extremity revascularization (TER). $\chi^2 = 39.44$; p = 0.013

Parameter	β-coefficient	β 95% CI	p-value	Hazard ratio	Hazard ratio 95% CI
Age (years)	–0.069	–0.13– –0.01	0.02	0.93	0.88–0.99
Gender (female/male)	0.89	–0.62–2.4	0.25	2.43	0.54–11.0
Smoking	–0.40	–1.25–0.46	0.36	0.67	0.29–1.58
Hypertension	0.10	–0.85–1.06	0.83	1.11	0.43–2.89
Diabetes mellitus	0.56	–0.53–1.67	0.31	1.76	0.59–5.26
Dyslipidemia	–3.08	–4.69– –1.48	0.0001	0.046	0.01–0.23
BMI	–0.09	–0.26–0.08	0.31	0.92	0.77–1.09
History of CAD	–0.08	–1.19–1.02	0.88	0.92	0.30–2.78
Past stroke	–0.91	–2.56–0.74	0.28	0.40	0.08–2.09
History of CHF	–0.32	–2.01–1.37	0.71	0.72	0.13–3.92
Creatinine > 2 mg/dL	1.48	–0.54–3.49	0.15	4.40	0.59–33.11
LDL cholesterol (mg/dL)	0.019	0.002–0.035	0.024	1.02	1.00–1.04
Triglycerides (mg/dL)	–0.002	–0.014–0.01	0.75	0.99	0.99–1.01
Leukocytes count (G/L)	0.001	–0.08–0.08	0.97	1.00	0.92–1.09
Hematocrit (%)	0.18	0.02–0.35	0.03	1.20	1.02–1.42
MCV (fL)	–0.06	–0.17–0.05	0.27	0.94	0.84–1.05
Platelets count (G/L)	–0.002	–0.01–0.002	0.28	0.99	0.99–1.00
MPV	–0.42	–0.82– –0.01	0.045	0.66	0.44–0.99
Creatinine (mg/dL)	1.85	–1.06–4.75	0.21	6.35	0.35–116.1
INR	0.46	0.12–0.79	0.01	1.58	1.13–2.21
aPTT	0.16	0.07–0.26	0.001	1.18	1.07–1.30
Fast glucose (mg/dL)	–0.001	–0.02–0.02	0.95	0.99	0.98–1.02

CI – confidence interval; BMI – body mass index; CAD – coronary artery disease; CHF – congestive heart failure; LDL – low-density lipoprotein; MCV – mean corpuscular volume; MPV – mean platelet volume; INR – international normalized ratio; aPTT – activated partial thromboplastin time.

worse outcomes after B, C and D classes of femoropopliteal lesion subintimal angioplasty with stenting.[18,19] Moreover, aggressive therapy with statins in patients with PAD significantly reduced all-cause mortality and the risk of stroke and showed a trend towards decreasing the risk of other cardiovascular end-points.[20] Statins also improved survival for 1 year after vascular surgery and demonstrated decreased incidence of lower extremity embolic complications after endovascular aortic repair.[21,22] In addition, several studies in recent years have shown that statins improved pain-free walking distance, ankle-brachial index and treadmill exercise time.[23,24] However, they did not affect long-term stent or bypass patency and limb salvage.[25]

In our study, a greater MPV value significantly decreased the risk of TER by approximately 34% (Table 3). In many investigations, an increase in MPV, but without a clearly defined cut-off value, was recognized as a marker of platelet renewal due to the baccelerated consumption in the thrombotic processes.[26,27] Larger platelets may potentially take part in acute cardiovascular events, including peripheral artery disease, unprovoked deep vein thrombosis, and pulmonary embolism, as well as in chronic ISR processes (growth factors accelerate neointima proliferation).[26–29] This data makes it difficult to explain our observations, which showed a favorable effect of greater MPV on TER risk with an average HR of 0.66 (Table 3).

Abnormalities in plasma coagulation factors are recognized mainly as a risk for venous thrombo-embolic disease. However, our investigation showed that longer blood coagulation times (greater aPTT, and INR) result in increased risk of TLR and TER (Table 2, 3). This suggests that decrease in blood coagulation had paradoxically unfavorable effect on treatment outcomes in patients with PAD. The effect of blood coagulation disorders on the course of PAD has been investigated in the past, but without finding a significant effect on intermittent claudication, and in the prevention of restenosis/reocclusion following peripheral endovascular treatment.[30,31]

Being aged over 45 in males and 55 in females is recognized as a risk factor for cardiovascular event and death.[16,17] In our study, paradoxically, older patients had a 7% decreased risk of TER (Table 3). However, in a recent study by Dippel et al., increased age was also found to decrease the occurrence of TLR (HR 0.77; 95% CI 0.61–0.96) when they compared the use of excimer laser atherectomy (ELA) with adjunctive percutaneous transluminal angioplasty (PTA) with PTA alone in a Cox proportional-hazards model.[3] It is worth underlining that in

this study, similar to our observations, diabetes mellitus was not an independent variable determining risk of TLR (Fig. 2). The negative correlation between age and TLR risk may be explained by greater atherosclerotic plaque stabilization, better collateral circulation development, and, possibly, a less invasive therapeutic attitude to older individuals.

As with most researchers authors, we could not avoid some methodological shortcomings that could have influenced the strength of the deductions based on our results. The main limitations of the paper are the retrospective type of analysis, the small sample size, the lack of inclusion of all peripheral artery stenting performed at our center, and the lack of a random selection of patients included in the analysis (potential selection bias). However, completion of these aforementioned requirements exceeded our abilities due to the great number of procedures introduced into our database (more than one thousand), and the lack of a complete follow-up for the treated patients. Moreover, the small number in the sample made it impossible to divide the patients according to the level of stenting performed, aortoiliac and femoropopliteal, which would certainly affect the risk of TLR and TER.[2] On the other hand, the limitations mentioned did not interfere with our ability to achieve statistical significance in the analyses undertaken, although our results, especially concerning their lipidal aspects, should be interpreted carefully. On the other hand, any doubts that have arisen show the need to perform more studies on hypolipidemic medication regarding standard end-points in patients with PAD after stenting.

Conclusions

Endovascular treatment with stenting in patients with atherosclerotic peripheral arterial disease is effective (70% of primary stent patency), especially as these procedures may be repeated and may postpone the need for a leg amputation.

Risk of TLR and TER was decreased to the greatest extent by the presence of dyslipidemia in spite of statins therapy.

The effect of dyslipidemia, hypolipidemic therapy, MPV, and main plasma coagulation tests (aPTT and INR) on the risk of TLR and TER need to be evaluated.

References

1. Laird JR, Yeo KK. The treatment of femoropopliteal in-stent restenosis: Back to the future. *J Am Coll Cardiol*. 2012;59:24–25.
2. Norgren L, Hiatt WR, Dormandy JA, et al. Inter-Society consensus for the management of peripheral arterial disease (TASC II). *J Vasc Surg*. 2007;45(Suppl S):S5–S67.
3. Dippel EJ, Makam P, Kovach R, et al. Randomized controlled study of excimer laser atherectomy for treatment of femoropopliteal in-stent restenosis: Initial results from the EXCITE ISR (EXCImer Laser Randomized Controlled Study for Treatment of FemoropopliTEal In-Stent Restenosis) Trial. *JACC Cardiovasc Interv*. 2015;8:92–101.
4. Bosiers M, Scheinert D, Peeters P, et al. Randomized comparison of everolimus-eluting versus bare-metal stents in patients with critical limb ischemia and infrapopliteal arterial occlusive disease. *J Vasc Surg*. 2012;55:390–398.
5. Singh GD, Armstrong EJ, Laird JR. Femoropopliteal in-stent restenosis: Current treatment strategies. *J Cardiovasc Surg (Torino)*. 2014;55:325–33.
6. Speck U, Scheller B, Hamm B. Drug-coated balloons for restenosis prophylaxis. *Rofo*. 2014;186:348–358.
7. Schmidt A, Zeller T, Sievert H, et al. Photoablation using the turbo-booster and excimer laser for in-stent restenosis treatment: Twelve-month results from the PATENT study. *J Endovasc Ther*. 2014;21:52–60.
8. Liistro F, Grotti S, Porto I, et al. Drug-eluting balloon in peripheral intervention for the superficial femoral artery: The DEBATE-SFA randomized trial (drug eluting balloon in peripheral intervention for the superficial femoral artery). *JACC Cardiovasc Interv*. 2013;6:1295–1302.
9. Tsetis D, Belli AM, Morgan R, et al. Preliminary experience with cutting balloon angioplasty for iliac artery in-stent restenosis. *J Endovasc Ther*. 2008;15:193–202.
10. Karthik S, Tuite DJ, Nicholson AA, et al. Cryoplasty for arterial restenosis. *Eur J Vasc Endovasc Surg*. 2007;33:40–43.
11. Tosaka A, Soga Y, Iida O, et al. Classification and clinical impact of restenosis after femoropopliteal stenting. *J Am Coll Cardiol*. 2012;59: 16–23.
12. Swanson N, Hogrefe K, Javed Q, Malik N, Gershlick AH. Vascular endothelial growth factor (VEGF)-eluting stents: In vivo effects on thrombosis, endothelialization and intimal hyperplasia. *J Invasive Cardiol*. 2003;15:688–692.
13. Grzelązka P, Koza K, Trofimiuk A, et al. The association between periprocedural factors and the late outcome of percutaneous stenting of lower extremity arteries. A retrospective cohort study. *Postep Kardiol Inter*. 2015;11(2);1–6.
14. Kavaliauskienė Z, Benetis R, Inčiūra D, Aleksynas N, Kaupas RS, Antuševas A. Factors affecting primary patency of stenting for TransAtlantic Inter-Society (TASC II) type B, C, and D iliac occlusive disease. *Medicina (Kaunas)*. 2014;50:287–294.
15. Diehm N, Baumgartner I, Jaff M, et al. A call for uniform reporting standards in studies assessing endovascular treatment for chronic ischaemia of lower limb arteries. *Eur Heart J*. 2007;28:798–805.
16. Perk J, De Backer G, Gohlke H, et al.; European Association for Cardiovascular Prevention & Rehabilitation (EACPR); ESC Committee for Practice Guidelines (CPG). European Guidelines on cardiovascular disease prevention in clinical practice (version 2012). The Fifth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (constituted by representatives of nine societies and by invited experts). *Eur Heart J*. 2012;33:1635–1701.
17. Montalescot G, Sechtem U, Achenbach S, et al. ESC guidelines on the management of stable coronary artery disease: The Task Force on the management of stable coronary artery disease of the European Society of Cardiology. *Eur Heart J*. 2013;34:2949–3003.
18. Gabrielli R, Rosati MS, Vitale S, et al. Randomized controlled trial of remote endarterectomy versus endovascular intervention for TransAtlantic Inter-Society Consensus II D femoropopliteal lesions. *J Vasc Surg*. 2012;56:1598–1605.
19. Baril DT, Marone LK, Kim J, Go MR, Chaer RA, Rhee RY. Outcomes of endovascular interventions for TASC II B and C femoropopliteal lesions. *J Vasc Surg*. 2008;48:627–633.
20. Antoniou GA, Fisher RK, Georgiadis GS, Antoniou SA, Torella F. Statin therapy in lower limb peripheral arterial disease: Systematic review and meta-analysis. *Vascul Pharmacol*. 2014;63:79–87.
21. Galiñanes EL, Reynolds S, Dombrovskiy VY, Vogel TR. The impact of preoperative statin therapy on open and endovascular abdominal aortic aneurysm repair outcomes. *Vascular*. 2014, pii: 1708538114552837.
22. Suckow BD, Kraiss LW, Schanzer A, et al. Statin therapy after infrainguinal bypass surgery for critical limb ischemia is associated with improved 5-year survival. *J Vasc Surg*. 2015;61:126–133.e1.
23. Markel A. Statins and peripheral arterial disease. *Int Angiol*. 2015;34:416–427.
24. Poredoš P, Jezovnik MK, Kalodiki Eet al. Medical management of patients with peripheral arterial disease. *Int Angiol*. 2015;34:75–93.

25. Dosluoglu HH, Davari-Farid S, Pourafkari L, Harris LM, Nader ND. Statin use is associated with improved overall survival without affecting patency and limb salvage rates following open or endovascular revascularization. *Vasc Med*. 2014;19:86–93.
26. Leader A, Pereg D, Lishner M. Are platelet volume indices of clinical use? A multidisciplinary review. *Ann Med*. 2012;44:805–816.
27. Pal R, Bagarhatta R, Gulati S, Rathore M, Sharma N. Mean platelet volume in patients with acute coronary syndromes: A supportive diagnostic predictor. *J Clin Diagn Res*. 2014;8:MC01–4.
28. Taglieri N, Saia F, Rapezzi C, et al. Prognostic significance of mean platelet volume on admission in an unselected cohort of patients with non ST-segment elevation acute coronary syndrome. *Thromb Haemost*. 2011;106:132–140.
29. Seyyed-Mohammadzad MH, Eskandari R, Rezaei Y, et al. Prognostic value of mean platelet volume in patients undergoing elective percutaneous coronary intervention. *Anadolu Kardiyol Derg*. 2015;15:25–30.
30. Cosmi B, Conti E, Coccheri S. Anticoagulants (heparin, low molecular weight heparin and oral anticoagulants) for intermittent claudication. *Cochrane Database Syst Rev*. 2014;5:CD001999.
31. Robertson L, Ghouri MA, Kovacs F. Antiplatelet and anticoagulant drugs for prevention of restenosis/reocclusion following peripheral endovascular treatment. *Cochrane Database Syst Rev*. 2012;8:CD002071.

Diagnosis and treatment of pelvic congestion syndrome: Single-centre experiences

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Abstract

Background. One of the underestimated causes of chronic pelvic pain (CPP) in women may be pelvic congestion syndrome (PCS) that is defined as the presence of varicose of ovarian and pelvic veins associated with chronic pain in the region of the pelvis. This pain is present longer than 6 months and intensifies with prolonged standing, coitus and menstruation. The disease constitutes a diagnostic as well as therapeutic problem, thus posing a challenge for the clinician. Transcatheter ovarian vein embolization might be a safe and effective option for PCS treatment.

Objectives. The objective of this study was to evaluate the efficacy of ovarian vein embolization ovarian as a method of the PCS treatment.

Material and methods. Between 2002–2012, 11 embolization procedures were performed in 10 women (age range: 34–43; median age 39) with the diagnosis of PCS. One patient underwent embolization procedure twice. In 1 case the combined therapy of endovascular embolization and surgical phlebectomy of vulvar varices was performed.

Results. There were no major intrainterventional complications. In all the patients (100%) a significant improvement in the clinical status was noted. The procedure improved the quality of life in the patients. Three women (30%) had a mild recurrence of the symptoms at mid-term follow-up. Among 8 women who had complained of dyspareunia prior to embolization 6 patients reported complete pain relief, in other 2 cases the pain subsided partially. There was a significant decrease in the severity of symptoms associated with hemorrhoids.

Conclusions. We consider embolization of insufficient ovarian veins an effective and safe way of treatment in a well-selected group of patients with PCS.

Key words: chronic pelvic pain, pelvic congestion syndrome, transcatheter embolization, ovarian vein insufficiency

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The syndrome of chronic pelvic pain (CPP) in women has been defined as "non-menstrual pain" of more than 6 months in duration.[1,2] Pain within the region of pelvis may be caused by many different conditions, thus making the diagnosis difficult. The incidence of CPP in women aged between 18 and 50 has been estimated as ca. 15%. It constitutes 10–40% of all outpatient gynecological visits.[2] In the USA, 35% of diagnostic laparoscopies and 15% of all hysterectomies have been performed because of the reported chronic pelvic pain.[1] The economic significance of the CPP cannot be underestimated: in the USA, in 15% of women with chronic pelvic pain, labor efficiency is reduced by ca. 14.8 working hours per month, thus causing the financial losses of ca. 14 billion dollars per year.[1,3] It is estimated that the total cost of medical care of chronic pelvic pain patients makes 39 billion dollars per year.[1,3–6]

There are many different conditions that can result in chronic pelvic pain. These might range from alimentary tract disorders through urological to gynecological diseases. Recent rapid advances in both diagnostic imaging modalities and interventional therapeutic procedures enhanced the ability of physicians to precisely diagnose and to treat effectively anatomical lesions responsible for chronic pelvic pain in most women.

Pelvic congestion syndrome (PCS) was first described by Richet in 1857 as a chronic dull pelvic pain caused by increased venous pressure, and the severity of the illness is associated with the number and tortuosity of dilated veins. The incidence of chronic pelvic pain is high; varicose veins have been observed in 10% of all women and 60% of them report CPP.[4] In spite of this relatively high incidence of CPP, PCS is correctly diagnosed in rare cases, possibly due to the limited diagnostic value of traditional examination methods. Pelvic congestion syndrome may present with a variety of other symptoms, e.g. dyspareunia, tender lower abdomen, vaginal discharge, vulvar swelling, neuropathy, rectal discomfort or frequent urination. Extensive pelvic varices are not only the cause of the CPP but potentially can also have a fatal outcome, due to a pulmonary embolism resulting from deep vein thrombosis.[5]

PCS results from pathological blood reflux seen typically in left ovarian vein, most frequently during pregnancy (Fig. 1).

The pathomechanism is similar as in varicose veins of the lower limbs and can be induced by hormonal changes as well as by the mechanical resistance and pressure. The cause of PCS may also be associated with anatomical abnormalities, such as compression of the left renal vein by the superior mesenteric artery (the nutcracker syndrome), its retroaortic course or a venous hypertension secondary to anomalies of the inferior vena cava (IVC). The literature provides for cases of pelvic varices caused due to compression of the left common iliac vein by the right common iliac artery (May-Thurner syndrome), which may result in venous thrombosis of the lower limbs (Fig. 2). Concomitant vulvar varicose veins may develop

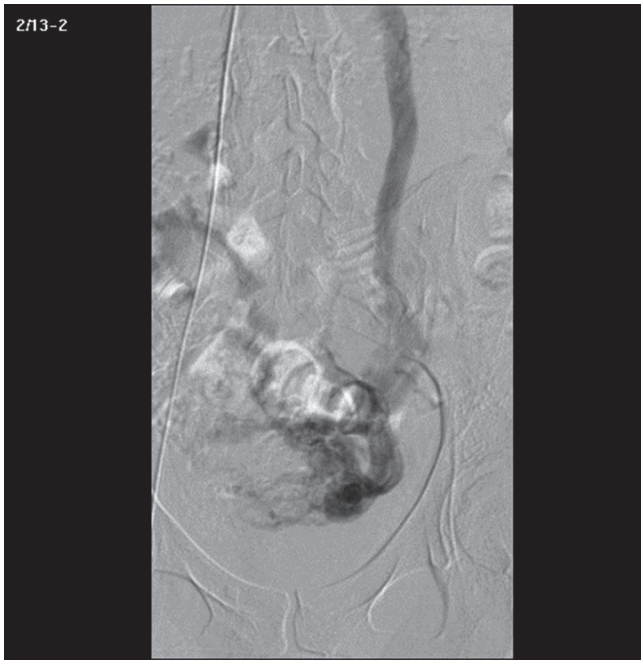

Fig. 1. Venogram demonstrating insufficient, dilated left ovarian vein

also externally through the great saphenous vein (GSV) and in particular through the collateral medial marginal vein, which is the junction between the venous circulation of the lower limb and the internal iliac vein (hypogastric vein) (Fig. 3). Some role may also be attributed to the vein that drains the muscles of the thigh, the branch that arises from the common femoral vein.

Most frequently, the female patients see a general practitioner or a surgeon because of the chronic pain in the lower abdomen, frequently associated with exertion.

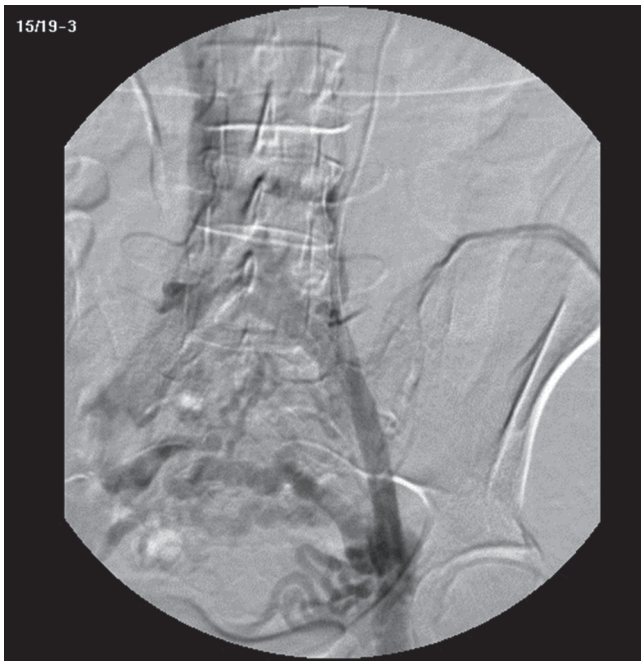

Fig. 2. Venogram presenting dilated left pelvic veins due to May-Thurner syndrome

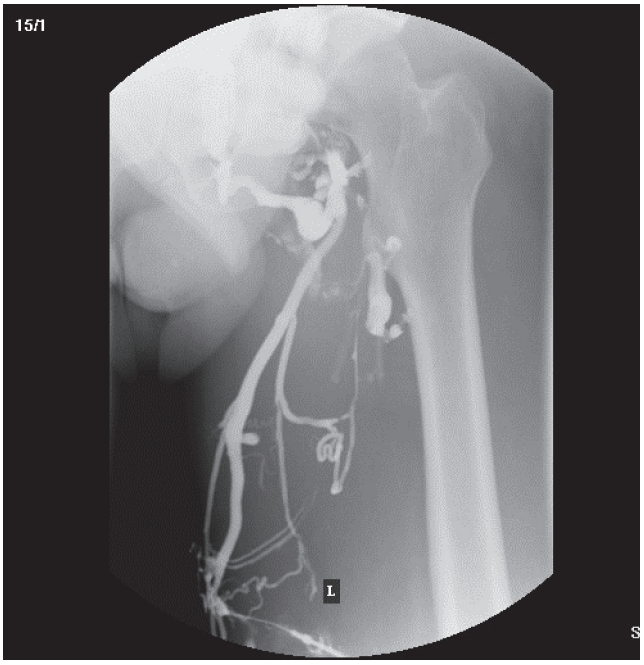

Fig. 3. Venogram demonstrating varicose veins of the left perineal region that communicate with varicosities within minor pelvis

A physical examination sometimes reveals that the tenderness in localized in the ovaries. Symptoms aiding the diagnosis might also be the lower extremities varicose veins disease, but, above all, the vulvar varices and to some lesser degree the hemorrhoidal varices.

Imaging methods are the most critical in the evaluation of pelvic varicose veins. The recently developed non-invasive or minimally invasive procedures, as the US (including Color Doppler, duplex-Doppler, Power Doppler imaging), CT or MRI techniques replaced the traditional phlebography in diagnosing the pelvic varices, with the transvaginal US imaging being a preliminary imaging modality.[7–10]

Traditional phlebography allows direct visualization of the tortuous and dilated ovarian veins and is still considered the gold standard reference for an accurate inpatient diagnosis of PCS. There are several methods for direct opacification of the dilated varicose veins including selective venous catheterization of the ovarian veins, injection of contrast medium into vulvar varices. These methods are rarely used now because they are invasive and expose the patients, especially those of child-bearing age, to irradiation.[7,11,12]

There are 3 diagnostic criteria for establishing the diagnosis of pelvic congestion: a tortuous pelvic vein with a diameter more than 4 mm, slow blood flow (about 3 cm/s), and the dilated communicating arcuate veins.[11] Pelvic varices are visualized as dilated, tortuous, opacified tubular structures around the uterus and the ovary, with a possible extension into the broad pelvic ligament. They can also involve the paravaginal venous plexus.[13]

Based on above mentioned clinical presentation and results of imaging modalities we established a diagnosis of PCS in a relatively small group of patients and qualified them for predominantly endovascular treatment. The purpose of our study was to evaluate the efficacy of ovarian vein embolization as a method of the treatment of PCS.

Material and methods

This retrospective analysis includes 10 multiparous women (n = 10) aged between 34 and 43 (mean age 39) with clinical suspicion of PCS that were referred to our institution in order to confirm the diagnosis and to undergo the treatment. All ten patients (100%) suffered from chronic pelvic pain. Eight women (80%) complained of dyspareunia and tender lower abdomen. Five women (50%) complained of rectal discomfort and/or frequent urination. Three of the 10 patients (30%) suffered from neuropathy of pubic region. In 2 patients (20%) vaginal discharge was observed.

The pre-hospitalization examinations allowed us to exclude some other potential conditions causing pelvic pain, especially those associated with the digestive system and endometriosis.

Non-invasive portion of diagnostic protocol of PCS consisted of both lower-extremity venous duplex scan and a transvaginal duplex scan. In the case of inconclusive or ambiguous results of the ultrasound scan, magnetic resonance imaging angiography (MRA) was additionally performed. Finally, the minimally invasive venography of pelvic veins was carried out.

Eleven procedures of ovarian vein (OV) embolization were carried out in 10 women under local anesthesia. Transfemoral approach was used in all the cases. After the puncture of the right femoral vein cobra catheter was advanced into the left renal vein (LRV). Subsequently, the left ovarian vein (OV) was catheterized and a venogram was obtained to identify the gonadal vein tributaries and pelvic collaterals. Venograms were obtained in a semi-upright position of the patients and during Valsalva maneuver. A selective venography of right OV was performed with direct cannulation from the IVC. The indication for embolization included the following: an OV diameter of more than 10 mm, moderate to severe congestion within the ovarian plexus, uterine venous engorgement, and filling of the contralateral pelvic veins. All the 10 cases were treated with a transcatheter coil embolization using Gianturco 0.035-inch MReye embolization coils (Cook Medical) of various diameter and various length depending on individual anatomical conditions. Three patients needed an additional embolization with the use of embolization glue – the mixture of histoacryl glue (n-butyl-2-cyanoacrylate – NBCA) (Histoacryl; B. Brown, Melsungen, Germany) with oil iodinated contrast medium (Lipiodol® UltraFluide; Guerbet, USA Guerbet) in concentration 17–25%. Next, a follow-up venogram was obtained to confirm both the

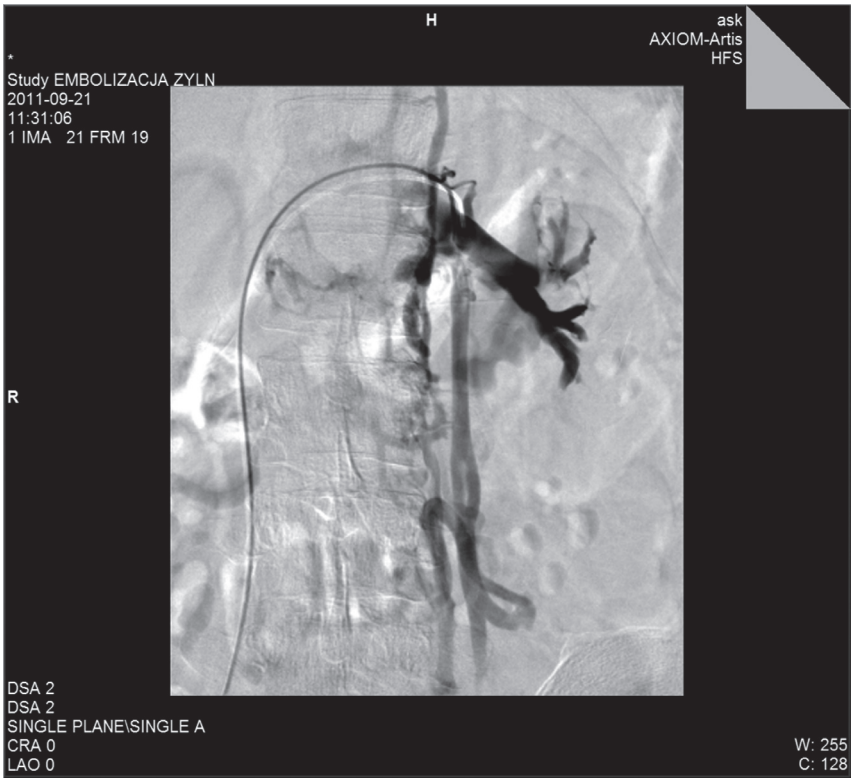

Fig. 4. Descending venography showing partially double insufficient ovarian vein

occlusion of OV and the concomitant parallel trunks as well as the patency of the LRV. Finally, the catheter was removed.

In 1 patient the 2nd procedure was performed 12 months following the first embolization due to the manifestation of accessory ovarian vein insufficiency. One patient underwent a combination of endovascular embolization and surgical phlebectomy of vulvar varices. The diagnosed pathology, the type of intervention and the results of the treatment are summarized in Table 1. The follow-up included an evaluation of a clinical presentation every 2 months and a Doppler-US examination was carried out in each patient every 6 months.

Results

The ovarian vein embolization was performed with no major general or local complications. Only in 1 case some embolization glue migrated into the pulmonary circulation, thus causing a clinically insignificant right lower-lobe embolism that was controlled immediately during the procedure. In 1 case, a paroxysmal tachyarrhythmia was observed during the procedure and it was controlled intraprocedurally without any long-term consequences.

In most of the cases, the ovarian vein embolization using coils proved sufficient and in 3 cases it was necessary to additionally use histoacrylic glue. In 1 case the

Table 1. Diagnosed pathology, type of intervention and results of treatment of patients with diagnosed PCS

No.	Pathology	Number of embolizations/ /embolization agent	Follow-up (months)	Clinical outcomes (regression of symptoms)
1.	insufficiency of left ovarian vein (OV)	1/coils	13.9	complete regression
2.	insufficiency of left OV	1/coils	22.5	significant regression
3.	insufficiency of left OV	1/coils	14	complete regression
4.	insufficiency of left OV	1/coils	26	complete regression
5.	insufficiency of left internal iliac vein and left OV	2/coils; coils and glue	16	significant regression
6.	insufficiency of left OV	1/coils	12	complete regression
7.	insufficiency of duplicated left OVs vulvar varicosities, lower limb varicosities	combined treatment: 3/surgery; coils; coils + glue	8	significant regression
8.	insufficiency of left OV	1/coils	13.9	complete regression
9.	insufficiency of left OV	1/coils	13.9	complete regression
10.	insufficiency of duplicated left OV	1/coils + glue	2	complete regression

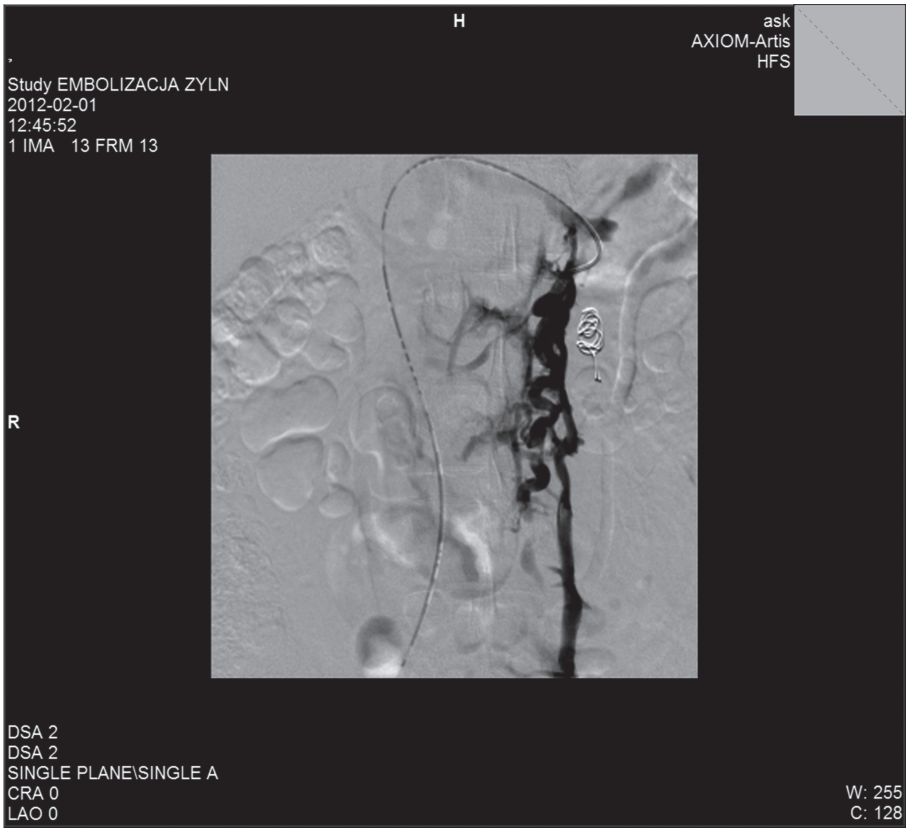

Fig. 5a. Second procedure with embolization of duplicated left ovarian vein

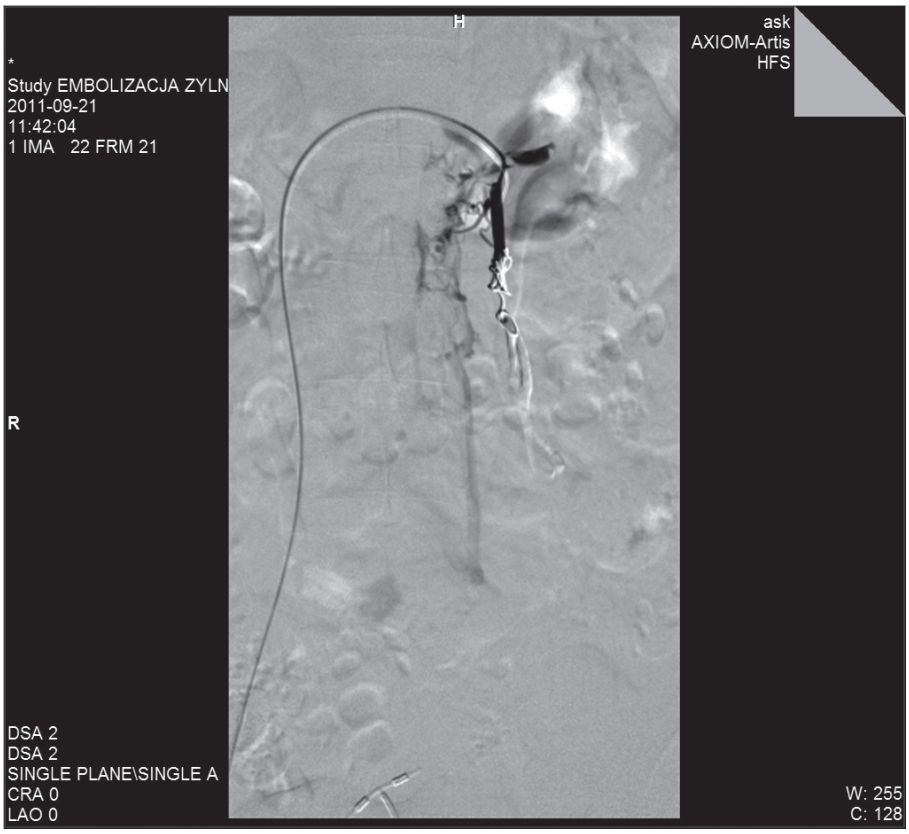

Fig. 5b. Coil embolization of the left ovarian vein

combination of surgical and endovascular approach was implemented. The 1st stage of the treatment included the surgical removal of varicose veins in the lower limbs and partially the vulvar varicose veins. The 2nd stage consisted of 2 embolizations of ovarian veins that appeared to be double, with one of them seemingly unchanged. Eventually, the additional sclerotherapy of vulvar varicosities was performed with the total pain relief.

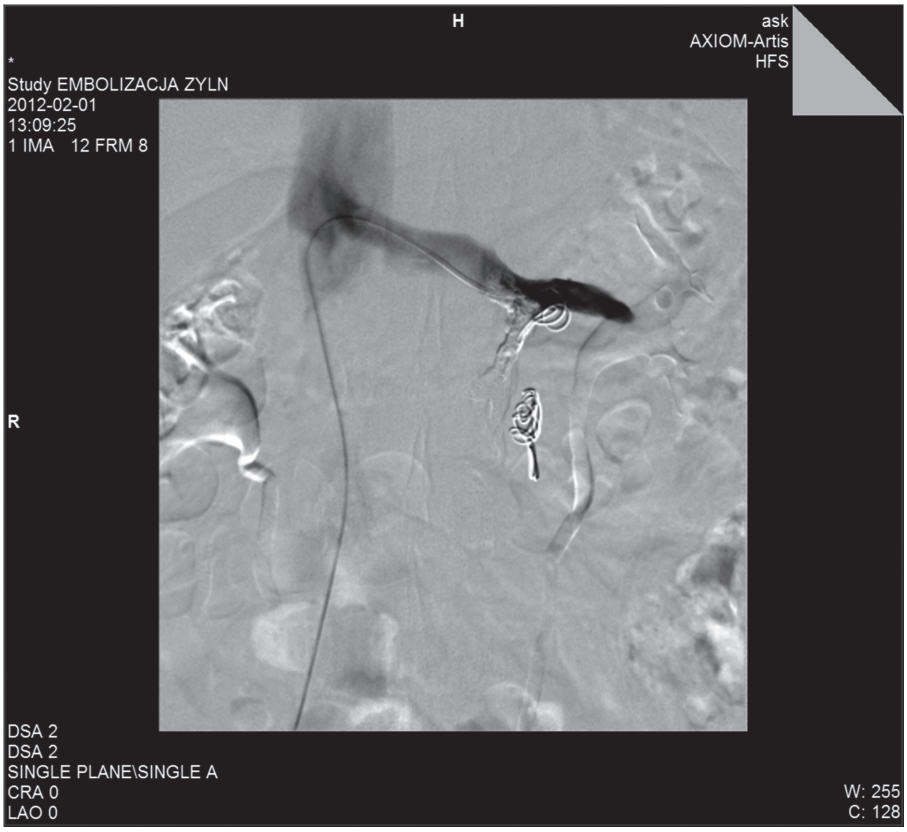

Fig. 5c. Postembolization follow-up venogram showing the proper patency of the left renal vein

We did not observe a contrast extravasation during the embolization procedure. There were no major morbidity rates and no mortality rate neither intrainterventionally nor during the follow-up.

The mean follow-up period amounted to 16.4 months (range: 2–26 months). In all the patients the significant improvement of the clinical status was observed. In 7 cases (70%) total pain relief was observed. In 3 women (30%) a mild recurrence of the symptoms was noted after 8–12 months postinterventionally; however, it was not necessary to repeat the embolization procedure in those patients after an initial 100% symptoms relief.

Among 8 women who had complained of dyspareunia prior to embolization, 6 patients reported complete pain relief, in other 2 cases pain subsided partially. There was a significant decrease in the severity of hemorrhoids associated symptoms.

Discussion

In 1950 Topolanski-Sierra coined the term of chronic pelvic congestion and demonstrated the relationship between chronic abdominal pain and pelvic varicose veins, and stressed the necessity of its treatment.[14] One of the suggested methods of treatment included chemical damage of the fallopian tubes by the administration of goserelin acetate.[15] In 1980, Rundquist described a new method of surgical treatment, with extraperitoneal ligation of the left ovarian vein. This method proved effective in the treatment of the lower abdominal pain.[16,17] It found many followers, especially in the context of the negative evaluation of hysterectomy. In 33% of patients after hysterectomy no relief of symptoms was observed and in 20% of patients symptoms reoccurred.[18–20] Overall, each kind of surgical approach is associated with the risk of anesthesia, laparotomy or laparoscopic treatment.

First introduced in 1993 by Edwards et al., the technique that truly revolutionized PCS treatment involves catheterization of the ovarian veins.[21] The procedure is usually performed during the diagnostic phlebography and it involves venous obliteration with a foam or embolization agent.

There is no data from randomized trials relating to PCS treatment. Several published results of trial series do not attempt to compare the embolization of incompetent pelvic veins with the other, alternative methods. Randomized trials are of particular significance especially when the main symptom of the disease is the pain, because pain is highly subjective. The aforementioned published trial results relate to the retrospective cases. The search of the literature on the subject has been conducted with the use of the MEDLINE database and covered the period to March 2012 included. The largest series of clinical trials and case studies discussed by various authors are listed in the Table 2.[25–30]

In addition to the evaluation of clinical efficacy of the embolization procedure for the treatment of PCS, attempts have been made to develop the uniform diagnostic criteria and to determine if the chronic pain is caused

Table 2. Outcomes of clinical trials trials

Study	Country	Number of patients	Mean follow-up (months)	Clinical outcomes (regression of symptoms)
Maleux et al., 2000[1]	Belgium	41	19.9	significant: 58.9%
Venbrux et al., 2002[2]	USA	56	22.1	significant or partial: 96%
Pieri et al., 2003[3]	Italy	33	12.0	significant: 100%
Kim et al., 2006[4]	USA	127	45.0	significant: 83%
Kwon et al., 2007[5]	Korea	67	~44.8	significant or partial: 82%
Gandini et al., 2008[6]	Italy	38	12.0	significant: 100%

solely by vascular lesions. The significance of B-mode US, duplex Doppler imaging, CT and magnetic resonance imaging for the differential diagnostic considerations has been stressed.[31,33] The authors note that the published material hardly suggests guidelines for the diagnosis of PCS; moreover, the definitions of symptoms and of pelvic pain vary greatly among studies. Furthermore, in the majority of trials no statistical methods have been applied for the objective presentation of the results. In his publication, Ball argues whether an acute pain in the lower abdomen is indeed caused by pelvic varices.[34] The author demonstrates that not all patients with vascular lesions suffer from pelvic pain.

So far there have been no unambiguous randomized trials or criteria that would qualify women for PCS treatment. However, the long history of research on the essence of this condition allowed for the development of effective and minimally invasive treatment for women with this chronic illness. There are many different conditions that can directly cause the main symptom: dull pain in the lower abdomen. Anatomic abnormalities, pregnancies, hormonal changes, body weight gain or the lower extremities varicose vein disease can all contribute to the unidirectional course and intensification of PCS. Venous valve incompetence can result in pelvic engorgement and resultant edema thus causing pelvic pain. This pain is exacerbated after prolonged sitting, at the end of a day, during or directly after sexual activity (dyspareunia), during menstruation. There are also other unspecific symptoms of varying intensity. Women affected with these symptoms can experience nausea, depression, tender lower abdomen, vaginal discharge, vulvar swelling, neuropathy, rectal discomfort or frequent urination. Most often affected women are in the pre-menopausal age; therefore, the suggested causes include the estrogen influence that significantly weakens venous walls. Pelvic vein thrombosis is a potentially fatal condition and is seen as a complication of the abovementioned condition.

Contemporary medical imaging techniques have broadened diagnostic procedures. Pelvic ultrasound scans, transvaginal US and CT have become the first imaging diagnostic procedures in patients with chronic pelvic pain. They all visualize the uterus and its vicinity. It is, however, the computed tomography with the use of contrast media that demonstrated the highest efficacy in visualizing the vascular lesions. Then again, the US in the duplex Doppler mode allows for the dynamic evaluation of the blood flow, the detection of reversed flow in the left ovarian vein. In chronic pelvic pain the transvaginal ultrasound can be especially useful for the preliminary diagnoses of tumors and cysts, and endometric cysts. However, the proper diagnosis is often missed because women lie down for a pelvic examination. In this position, the ovarian veins will not fill enough with blood to reveal the vascular changes. It should be remembered that a standard transvaginal US will not reveal vascular changes and is reliable only when duplex Doppler modality is implemented.

In their advanced diagnostic management most diagnosticians rely on MRI. This procedure can be carried out as an outpatient procedure, it is non-invasive, does not require ionizing radiation and is of particular value in diagnosing the pelvic varicose veins. Typical findings in patients with PCS on MRI include dilated, enhancing tubular structures near the ovaries and uterus, with possible extension into the broad pelvic ligament. As indicated by the clinical experience, MRI has become the most valuable diagnostic modality, particularly in T1-weighted scans and above all with volumetric 3D imaging following intravenous injection of gadolinium.

Traditional phlebography still seems to be the golden standard reference for the best visualization of the venous circulation and above all it allows for therapeutic procedures. However, it should be remembered that it is an invasive procedure and, therefore, should be undertaken after appropriate non-invasive demonstration of lesions as both a diagnostic and therapeutic procedure. In this procedure, it is possible to plan and perform the occlusion of insufficient veins.

Laparoscopy is the most commonly used diagnostic technique used in women with chronic pelvic pain. This direct visualization is an excellent tool to exclude other pelvic pathologic conditions such as, e.g. endometriosis. It is, however, not useful for varicose vein diagnosis because it requires women to lie down for the procedure and involves insufflations of carbon dioxide that conceals pathological vessels and, as a rule, yields unsatisfactory results.

In our material the patients reported substantial (30%) or complete relief (70%) of their pain following trans-

catheter ovarian vein embolization. In the present study, 8 (80%) patients underwent only one embolization. One patient required a multistage treatment, involving embolization of ovarian veins and varicose veins surgery (including vulvar varicosities). There was significant relief of the following symptoms after embolization: pelvic pain; pain prior to and during menstruation, pain during and after sexual activity and moderate reduction of complaints that were associated with maintaining a standing position. The procedures performed did improve the patient's quality of life. No major local or general complications were observed. The present study was based on a limited number of patients, so all the results have to be interpreted with caution. However, there is still a lack of randomized controlled trials estimating ovarian vein embolization as a treatment of choice of PCS. Despite the limitations of our study we believe that venography with ovarian vein embolization could be the optimal therapeutic approach for pelvic congestion syndrome.

References

1. APGO Educational Series on Women's Health Issues. Chronic pelvic pain: An integrated approach Crofton, Md: APGO, January 2000.
2. Harris R, Holtzman S, Poppe A. Clinical outcome in female patients with pelvic pain and normal pelvic US findings. *Radiology*. 2000;216: 440–443.
3. Francica G, Giardiello C, Angelone G, et al. Abdominal wall endometriomas near cesarean delivery scars. *J Ultrasound Med*. 2003;22: 1041–1047.
4. Kuligowska E, Deeds L, Lu K. Pelvic pain: Overlooked and underdiagnosed gynecologic conditions. *Radiographics*. 2005;25(1):3–20.
5. Waliszewski P. Masywny zator tętnicy płucnej w przebiegu zakrzepicy splotów żylnych miednicy mniejszej. *Przew Lek*. 2000;9:105–108.
6. Belenky A, Bartal G, Atar E, et al. Ovarian varices in healthy female kidney donors: Incidence, morbidity, and clinical outcome. *Am J Roentgenol*. 2002;179(3):625–627.
7. Coakley F, Varghese S, Hricak H. CT and MRI of pelvic varices in women. *J Comput Assist Tomogr*. 1999;23:429–434.
8. Capasso P, Simons C, Trotteur G, et al. Treatment of symptomatic pelvic varices by ovarian vein embolization. *Cardiovasc Intervent Radiol*. 1997;20:107–111.
9. Tarazov P, Prozorovskij K, Ryzhkov V. Pelvic pain syndrome caused by ovarian varices: Treatment by transcatheter embolization. *Acta Radiol*. 1997;38:1023–1025.
10. Gargiulo T, Mais V, Brokaj L, et al. Bilateral laparoscopic transperitoneal ligation of ovarian veins for treatment of pelvic congestion syndrome. *J Am Assoc Gynecol Laparosc*. 2003;10:501–504.
11. Beard R, Highman J, Pearce S, et al. Diagnosis of pelvic varicosities in women with chronic pelvic pain. *Lancet*. 1984;2:946–949.
12. Maleux G, Stockx L, Wilms G, et al. Ovarian vein embolization for the treatment of pelvic congestion syndrome: Long-term technical and clinical results. *J Vasc Interv Radiol*. 2000;11:859–864.
13. Desimpelaere J, Seynaeve P, Hagers Y, et al. Pelvic congestion syndrome: Demonstration and diagnosis by helical CT. *Abdom Imaging*. 1999;24:100–102.
14. Topolanski-Sierra R. Pelvic phlebography. *J Obstet Gynecol*. 1958; 76(1):44–52.
15. Rundqvist E, Sandholm L, Larsson G. Treatment of pelvic varicosities causing lower abdominal pain with extraperitoneal resection of the left ovarian vein. *Ann Chir Gynaecol*. 1984;73:339–341.
16. Soysal M, Soysal S, Vidcan K, et al. A randomized controlled trial of goserelin and medroxyprogesterone acetate in the treatment of pelvic congestion. *Reprod Hum*. 2001;16(5):931–939.
17. Hiromura T, Nishioka T, Nishioka S, et al. Reflux in the left ovarian vein: Analysis of MDCT findings in asymptomatic women. *Am J Roentgenol*. 2004;183(5):1411–1415.
18. Beard R, Kennedy R, Gangar K, et al. PubMed Bilateral oophorectomy and hysterectomy in the treatment of intractable pelvic pain associated with pelvic congestion. *Br J Obstet Gynaecol*. 1991;98(10): 988–992.
19. Carter J. Review Surgical treatment for chronic pelvic pain. *JSLS*. 1998;2(2):129–139.
20. Takeuchi K, Mochizuki M, Kitagaki S. Laparoscopic varicocele ligation for pelvic congestion syndrome. *Int J Gynaecol Obstet*. 1996; 55:177–178.
21. Edwards R, Robertson J, MacLean A, et al. Case report: Pelvic pain syndrome- successful treatment of a case by ovarian vein embolization. *Clin Radiol*. 1993;47:429–431.
22. Kim H, Malhotra A, Rowe P, Lee J, Venbrux A. Embolotherapy for pelvic congestion syndrome: Long-term results. *J Vasc Interv Radiol*. 2006;17:289–297.
23. Nascimento A, Mitchell D, Holland G. Ovarian veins: Magnetic resonance imaging findings in an asymptomatic population. *J Magn Reson Imaging* 2002;15(5): 551–556.
24. Ignatio E, Dua R, Sarin S, et al. Pelvic congestion syndrome: Diagnosis and treatment. *Semin Intervent Radiol*. 2008;25(4):361–368.
25. Rozenblit A, Ricci Z, Tuvia J, et al. Incompetent and dilated ovarian veins: A common CT finding in asymptomatic parous women. *Am J Roentgenol*. 2001;176(1):119–122.
26. Venbrux A, Chang A, Kim H. Pelvic congestion syndrome (pelvic venous incompetence): Impact of ovarian and internal iliac vein embolotherapy on menstrual cycle and chronic pelvic pain. *J Vasc Interv Radiol*. 2002;13:171–178
27. Pieri S, Agresti P, Morucci M. Percutaneous treatment of pelvic congestion syndrome. *Radiol Med*. 2003;105:76–82.
28. Kim H, Malhotra A, Rowe P, et al. Embolotherapy for pelvic congestion syndrome: Long-term results. *J Vasc Interv Radiol*. 2006;17:289–297.
29. Kwon S, Oh J, Ko K, et al. Transcatheter ovarian vein embolization using coils for the treatment of pelvic congestion syndrome. *Cardiovasc Intervent Radiol*. 2007;30(4):655–661.
30. Gandini R, Chiocchi M, Konda D, et al. Transcatheter foam sclerotherapy of symptomatic female varicocele with sodium-tetradecyl-sulfate foam. *Cardiovasc Intervent Radiol*. 2008;31(4):778–784.
31. Naoum J. Endovascular therapy for pelvic congestion syndrome. *Methodist Debakey Cardiovasc J*. 2009;5(4):36–38.
32. Kies D, Kim H. Pelvic congestion syndrome: A review of current diagnostic and minimally invasive treatment modalities. *Phlebology*. 2012;27(Suppl 1):52–57.
33. Tu F, Hahn D, Steege J. Pelvic congestion syndrome-associated pelvic pain: A systematic review of diagnosis and management. *Obstet Gynecol Surv*. 2010;65(5):332–340
34. Ball E, Khan K, Meads C. Does pelvic congestion syndrome exist and can it be treated? *Acta Obstet Gynecol Scand*. 2012;91:525–528.

Serum carnitine and acyl-carnitine in patients with meningitis due to tick-borne encephalitis virus infection

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Abstract

Background. Hard ticks are the main vectors of tick-borne encephalitis virus (TBEV). Free carnitine (FC) and acylcarnitines (AC) have the basic role in β-oxidation as well as the modulation of immune and nervous system. Homeostasis of carnitines in the TBE patients was not studied so far.

Objectives. This study aimed to evaluate FC and AC serum concentrations in patients with meningitis due to TBEV infection before and after 14 ± 3 days of treatment.

Material and methods. The study was performed in 14 patients aged 48 ± 29 years that were divided a posteriori (based on their FC level before and after treatment) into 2 subgroups: 1–8 and 9–14. Diagnosis was based on the neurological, serological and pleocytosis evaluation.

Results. The FC level in patients 1–8 before treatment (24.1 ± 8.1) was significantly lower than in patients post-treatment (34.4 ± 8.3), lower than in the control group (40.5 ± 7.6), and lower than in patients 9–14 before treatment (40.0 ± 13.5) but not lower than in the patients 9–14 after treatment (24.7 ± 7.3 µmol/L), respectively, $p < 0.05$. AC concentration in the patients 1–8 before treatment (4.7 ± 2.2) was apparently lower than in patients post-treatment (9.5 ± 3.9 µmol/L) but the values were not significantly different. In patients 9–14 before treatment the AC concentration (16.3 ± 12.6) was higher than in patients after treatment (5.3 ± 4.0 µmol/L), but the difference was not statistically significant.

Conclusions. FC and AC homeostasis in circulation was disturbed in the patients with meningitis due to TBEV infection patients. The mean levels of FC and AC in 60% of the patients were below the normal range but normalized after treatment whereas in 40% of the patients they were near or at a normal range and significantly decreased after treatment. Explanation of this intriguing finding and its clinical significance is not easy without further studies.

Key words: carnitine deficiency, tick-borne encephalitis, free carnitine, acyl-carnitine

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Introduction

Tick-borne encephalitis (TBE) RNA virus (TBEV) from the *Flavivirus* genus belongs to the neurotropic viruses. Infection with the TBEV is initiated by a bite from an infected hard tick (i.e. *Ixodes ricinus*). The virus is present in the tick's saliva where there is a natural anesthetic; therefore, the tick bite may be unnoticed. The ticks may transmit more than one pathogen, thus complicating diagnosis and treatment of the TBE.[1] In 65–70% of TBEV infected humans, the virus does not cause any symptoms but in symptomatic patients the disease develops in 2 stages. First signs may include high temperature, headache, tiredness, muscle pain, which last 1–8 days after which most of the patients recover. In the second phase, the brain and spinal cord become affected by the virus that manifests in: meningitis, meningoencephalitis or meningoencephalomyelitis.[2,3]

Free carnitine (FC) and acylcarnitines (AC) are important, multifactorial substances. The majority of the FC originates from the diet, whereas about 25% is synthetized de novo from lysine and methionine in the liver, kidney and brain but not in the skeletal and cardiac muscles. FC provides fatty acids as well as the products of their peroxisomal partial oxidation into the mitochondria for the β-oxidation.[4,5] It removes an excess of toxic acyl-CoA from mitochondrial matrix, regulates acyl-CoA/free CoA ratio, and supplies acetyl groups for the synthesis of acetylcholine and for acetylation of nuclear histones in the central nervous system.[6] "Secondary" roles of carnitine include its actions as an immune system, gene and protein modulator, antioxidant, anti-inflammatory and anti-apoptotic factor.[7] Low serum carnitine levels were shown in patients with impaired immune reactions, metabolic disorders, recurrent infections, and chronic fatigue syndrome.[4,5,8] Carnitine deficiency may result from the malnutrition, malabsorption, peritoneal dialysis, and increased urinary AC excretion.[5,9] FC and AC may have an unexplored role in the pathophysiology of the tick-borne encephalitis resulting from TBE virus infection. Therefore, we aimed to evaluate serum concentration of the FC and AC in adult patients with meningitis resulting from TBEV infection, before and after supportive and symptomatic treatment.[8] Their homeostasis in the TBE patients was not studied so far.

Material and methods

Patients

The study group included 14 adults (6 male, 8 female) aged 48 ± 29 years with meningitis resulting from TBEV infection. Diagnosis was based on the neurological evaluation and serological determinations in the sera and cerebrospinal fluid (CSF). Upon admission all the patients exhibited typical symptoms seen in the TBE virus infection: fever lasting 1–3 weeks, headache, arthralgia, myalgia. Supportive and symptomatic treatment of the patients lasted 14 ± 3 days. The study group was divided a posteriori (based on the results of the FC determinations before and after treatment) into 2 subgroups: patients number 1–8 (4 male, 4 female) aged 44 ± 18 years, in whom the FC concentration increased as a result of treatment and patients number 9–14 (2 male, 4 female) aged 51 ± 19 years, in whom the FC concentration decreased after treatment. Serological and biochemical characteristics of both subgroups are shown in Table 1. Exclusion criteria: primary carnitine deficiency, patients with borreliosis, other recent viral infections, genetic and metabolic disorders, heart, renal and hepatic failure. The control group consisted of 32 healthy male (n = 17) and female (n = 15) aged 42 ± 11 years.

Table 1. Cerebrospinal fluid (CSF) and serum parameters in the TBE patients exhibiting increase (subgroup 1–8) or decrease (subgroup 9–14) of free carnitine (FC) concentration after 14 ± 3 days of treatment

Biological fluid	Parameters	Subgroup 1–8		Subgroup 9–14		Reference values
		before treatment	after treatment	before treatment	after treatment	
		mean ± SD				
CSF	total protein	67.6 ± 12.9	66.7 ± 45.9	56.7 ± 18.2	44.2 ± 3.4	15–45 mg/dL
	albumin	43.0 ± 9.8	24.4 ± 10.9	41.3 ± 12.8	nd	10–30 mg/dL
	glucose	56.8 ± 5.9	60.4 ± 11.3	62.0 ± 7.7	49.5 ± 0.5	48–85 mg/dL
	chloride	119 ± 2.6	118 ± 1.4	113 ± 1.1	117 ± 1.5	120–130 mM/L
	pleocytosis	112 ± 52.4	34.8 ± 19.8	58.8 ± 28.8	22.0 ± 2.0	< 5 cells/μL
		median (range)				
	IgM	30.2 (2.1, 147)	nd	1.9 (1.8, 351)	nd	antibody index < 1.4
	IgG	29.8 (6.0, 75.0)	nd	11.6 (0.0, 87.0)	nd	cut-off < 10 U/mL
Serum	IgM	45.7 (6.5, 207)	nd	9.3 (8.1, 500)	nd	antibody index < 1.4
	IgG	56.3 (19.1, 156)	nd	79.2 (22.0, 115)	nd	cut-off < 10 U/mL

Data are mean ± SD or median and range; nd – not determined.

Methods

Serological determinations

IgM and IgG antibodies against TBEV in the serum and CSF samples were assayed using ELISA Enzygnost Anti-TBE/FSME Virus [IgG, IgM] kit from Siemens, Marburg, Germany. Performance of the assay and interpretation of the data was done according to the Manufacturer's instruction. The data consistently indicated recent TBEV infection in our patients.

Carnitine assay

Free (FC) and total (TC) carnitine was determined in duplicates as previously detailed.[9,10] FC was measured in the serum filtrates without hydrolysis of acyl-carnitines, based on the reaction of FC with acetyl-CoA and carnitine acetyltransferase. TC concentration was quantified in the serum filtrates after hydrolysis of the carnitine esters: TC – FC = AC. Sensitivity of the method was 4.0 μmol/L. Inter- and intra-assay coefficient of variation (CV%) was 4.0 and 7.0%, respectively.

Statistical analysis

Data is expressed as mean ± SD or median (range) and was analyzed with STATISTICA (v. 8.0, StatSoft, Poland), using the Student t-test for independent samples. The normality of the variables distribution was tested using Shapiro-Wilk statistics and normality plots. Differences between 2 independent groups were analyzed using U Mann-Whitney non-parametric test. Differences between patients before and after treatment were tested using the Wilcoxon signed rank test. Statistical significance was assumed at $p < 0.05$.

Results

Serum concentrations of FC measured before and after 14 days of treatment in 14 patients with meningitis resulting from TBE virus infection are shown in Fig. 1. In the patients 1–8 the FC levels increased, whereas in the patients 9–14 they decreased after treatment. Before treatment, FC levels in patients 1–8 and 10, 13 were below normal range, whereas in patients 9, 11, 12, 14 within the normal range.

Mean concentrations of the FC and AC are shown in Table 2. Mean FC levels in the patients 1–8 before treatment (24.1 ± 8.1 μmol/L) were significantly lower ($p < 0.05$) than in patients post-treatment (34.4 ± 8.3 μmol/L), and lower than in healthy controls (40.5 ± 7.6 μmol/L), lower than in patients 9–14 before treatment (40.0 ± 13.5 μmol/L), but not lower than in patients 9–14 post-treatment (24.7 ± 7.3 μmol/L), $p > 0.05$. As a result of treatment, FC level in the patients 1–8 reached the values seen in healthy controls (Table 2). In contrast, FC level in patients 9–14 before treatment was the same (40.9 ± 13.5 μmol/L) like in the control group (40.5 ± 7.6 μmol/L) and statistically significantly decreased (24.7 ± 3.7 μmol/L) after treatment ($p < 0.05$) (Table 2).

Mean AC concentration in the patients 1–8 before treatment (4.7 ± 2.2 μmol/L) was apparently lower than that after treatment (9.5 ± 3.9 μmol/L), but the difference did not reach statistical significance, although it was significantly lower than that seen in healthy controls (13.5 ± 8.4 μmol/L, $p < 0.001$) (Table 2). In the patients 9–14 before treatment the AC concentration (16.3 ± 12.5 μmol/L) was apparently higher than that seen

Table 2. Mean serum free carnitine (FC) and acylcarnitine (AC) concentration in the patients from subgroups 1–8 and 9–14 with meningitis due to tick-borne encephalitis virus (TBEV) infection before and after treatment

Group	Patients	FC (μmol/L)	AC (μmol/L)
(I)	control group	40.5 ± 7.6	13.5 ± 8.4
	TBE patients		
(II)	1–8 (before)	24.1 ± 8.1	4.7 ± 2.2
(III)	1–8 (after)	34.4 ± 8.3	9.5 ± 3.9
(IV)	9–14 (before)	40.9 ± 13.5	16.3 ± 12.5
(V)	9–14 (after)	24.7 ± 3.7	5.3 ± 4.0
	p-values		
	II vs I	< 0.000	< 0.007
	III vs I	ns	ns
	IV vs I	ns	ns
	V vs I	< 0.000	< 0.001
	II vs III	< 0.011	ns
	IV vs V	< 0.027	ns
	II vs IV	< 0.016	ns
	II vs V	ns	ns
	III vs IV	ns	ns
	III vs V	< 0.045	< 0.033

values are mean ± SD; ns – not significant; vs – versus.

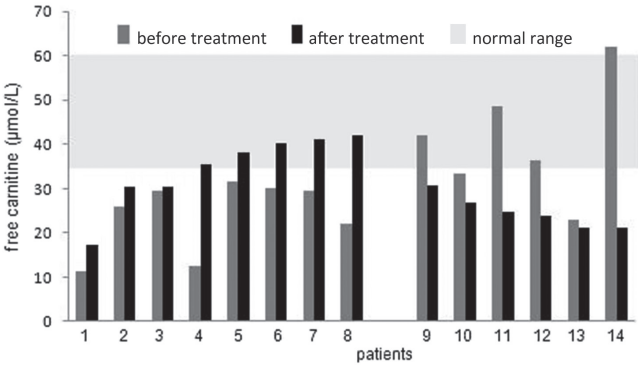

Fig. 1. Serum free carnitine in patients with meningitis due to tick-borne encephalitis virus infection before and after 14 days of treatment

after treatment (5.3 ± 4.0 μmol/L), but the difference was not statistically significant (p > 0.05), Table 2.

In the patients 1–8, IgM index (positive > 1.4) and IgG level (cut-off 10 standard units, U/mL) at admission was (median and range) 45.7 (6.5, 207) and 56.3 (19.1, 156), and in the CSF 30.2 (2.1, 147) and 29.8 (6.0, 75.0), respectively. In the patients 9–14 IgM index and IgG concentration (U/mL) was 9.3 (8.1, 500) and 79.2 (22.0, 115), and in the CSF 1.9 (1.8, 351) and 11.6 (0.0, 87.0), respectively. Pleocytosis (cut-off < 5 cells/μL) in the patients 1–8 at admission was 112 ± 52.4 cells/μL and after treatment: 34.8 ± 19.8 cells/μL, whereas in the patients 9–14 it was 58.8 ± 28.8 cells/μL and 22.0 ± 2.0 cells/μL, respectively. There were no statistically significant differences between biochemical parameters, IgG and IgM titers and pleocytosis between subgroups as well as between subgroups before and after treatment, respectively (Table 1).

Discussion

In this preliminary, observational study we have shown that FC and AC homeostasis in circulation is disturbed in the patients with meningitis due to TBEV infection. Supportive and symptomatic treatment of the patients and/or their natural healing process had an impact on the carnitine levels. Upon admission, the majority of our patients exhibited a substantial deficiency of the serum FC. Energy-dependent immunological mobilization of the infected organism (synthesis of the IgG/IgM anti-TBEV antibodies, anti- and pro-inflammatory cytokines) and decreased food intake may be responsible for the lowering of carnitines in circulation and tissues.[4,5] Normal availability of FC is crucial for the efficient β-oxidation, generation of AC and supply of the energy. Although glucose, not fatty acids, is a major source of energy in the brain, it has been shown that FC and AC deficiency (i.e. due to starvation) may cause metabolic encephalopathy.[8] About 99% of the carnitines are present inside the cells, but it is believed that circulating fraction of FC, TC and AC reflects their homeostasis in whole organism.[5]

Although all the patients were recovering well from the disease, we have noticed 2 types of patients. FC and AC serum levels in patients 1–8 prior to treatment were below the normal range, and significantly increased as a result of treatment. In contrast, FC and AC levels in patients 9–14 prior to treatment were significantly higher than those in the patients 1–8 before treatment and decreased below the normal range after treatment (Fig. 1, Table 2). Explanation of this intriguing finding is not easy without further basic and clinical studies on a numerous groups of patients.

Due to the small number of patients, it was not possible (or allowed) to perform an analysis of the correlations between carnitine concentrations and biochemical parameters, pleocytosis and IgM and IgG titers. It is tempting to speculate that differences in metabolic rate and immune system among the patients may be responsible for the observed phenomenon.

Some symptoms that accompany various clinical types of the TBE may co-result from the deficiency of carnitines. Carnitines supplementation has been shown to improve the overall energy status of the brain and the whole organism and to ameliorate symptoms of various diseases including neurological disturbances.[7] However, this topic also requires further studies.

References

1. Lani R, Moghaddam E, Haghani A, Chang LY, AbuBakar S, Zandi K. Tick-borne viruses: A review from the perspective of therapeutic approaches. *Ticks Tick Borne Dis.* 2014;5:457–465.
2. Holzmann H. Diagnosis of tick-borne encephalitis. *Vaccine.* 2003; 21 Suppl 1:36–40.
3. Lindquist L, Vapalahti O. Tick-borne encephalitis. *Lancet.* 2008;371: 1861–1871.
4. Famularo G, De Simone C, Trinchieri V, Mosca L. Carnitines and its congeners: A metabolic pathway to the regulation of immune response and inflammation. *Ann N Y Acad Sci.* 2004;1033:132–138.
5. Flanagan JL, Simmons PA, Vehige J, Willcox MD, Garrett Q. Role of carnitine in disease. *Nutr Metab (Lond).* 2010;7:30–34.
6. Madiraju P, Pande SV, Prentki M, Madiraju SR. Mitochondrial acetylcarnitine provides acetyl groups for nuclear histone acetylation. *Epigenetics*. 2009;4:399–403.
7. Ribas GS, Vargas CR, Wajner M. L-carnitine supplementation as a potential antioxidant therapy for inherited neurometabolic disorders. *Gene*. 2014;533:469–476.
8. Jones LL, McDonald DA, Borum PR. Acylcarnitines: Role in brain. *Prog Lipid Res*. 2010; 49:61–75.
9. Kępka A, Minarowska A, Minarowski Ł, et al. Serum and urinary carnitine in children with cystic fibrosis. *Prog Health Sci.* 2013;3:13–18.
10. Cederblad G, Harper P, Lindgren K. Spectrophotometry of carnitine in biological fluids and tissue with a Cobas Bio centrifugal analyzer. *Clin Chem.* 1986;322:342–346.

Cardiac tumors in children: A 20-year review of clinical presentation, diagnostics and treatment

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D – writing the article; E – critical revision of the article; F – final approval of article

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Abstract

Background. The use of new imaging techniques has contributed significantly to earlier diagnosis and treatment of cardiac tumors.

Objectives. The aim of the study was to analyze data from children with cardiac tumors in terms of clinical presentation, the role of noninvasive diagnostic procedures and the long-term outcome.

Material and methods. The data analyzed retrospectively concerned 30 children in whom cardiac tumors were diagnosed from January 1995 to July 2015. The cardiac evaluation included a review of the subjects' medical records and medical history, a physical examination, standard 12-lead electrocardiography, echocardiography and 24-h Holter ECG monitoring at the time of diagnosis and at 6–12 month intervals during the follow-up at the authors' outpatient clinic.

Results. Most of the children did not need cardiac surgery; surgical tumor excision was necessary in 3 cases only. There was 1 death in the follow-up period. Rhabdomyoma was diagnosed in 22 cases, and in 16 of them tuberous sclerosis was confirmed during the follow-up period. In the remaining 8 cases, fibroma was the most likely diagnosis.

Conclusions. The symptomatology of cardiac tumors in children can vary greatly, from the absence of any symptoms up to heart failure and respiratory distress indicating the need for surgical intervention. The diagnosis of cardiac tumors relies almost exclusively on noninvasive imaging techniques. The observations in this study confirm the fact that the most common cardiac tumor in children is rhabdomyoma, which may disappear spontaneously. Most patients with cardiac tumors do not require treatment.

Key words: tumor, cardiac, pediatric, fetal

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It is still difficult to estimate the incidence of cardiac tumors in the pediatric population.[1–3] Most heart tumors are benign, and rhabdomyoma is the most common of them.[1,2] Malignant and metastatic tumors have been described, but they are rare. Cardiac tumors are mainly manifested by murmurs, arrhythmias, cyanosis, respiratory distress, hemopericardium or exudative pericarditis and heart failure.[1,2] The use of new imaging techniques has contributed significantly to earlier diagnosis and treatment.[1–3]

The aim of this study was to analyze data from children with cardiac tumors in terms of the clinical presentation, the role of noninvasive diagnostic procedures and the long-term outcome.

Material and methods

The data analyzed retrospectively concerned 30 children in whom cardiac tumors were diagnosed from January 1995 to July 2015 (Table 1) at the authors' clinic. The cardiac evaluation included a review of the subjects' medical records and medical history, a physical examination, standard 12-lead electrocardiography, echocardiography and 24-h Holter ECG monitoring at the time of diagnosis and at 6–12 month intervals during the follow-up period. As there is no uniform heart failure functional classification for children, the present authors decided to use the Ross classification (or a modification thereof) for children below 14 years of age and the New York Heart Association (NYHA) functional classification for children aged 14 or over.

From 1995 to 2005, echocardiography was the only noninvasive imaging tool available at the clinic. Since 2005 cardiac computed tomography (CT) has also been available, since 2010 magnetic resonance imaging (MRI) as well.

Because of the great progress in prenatal diagnosis, since 2007 it has been possible to diagnose children with a positive family history of tuberous sclerosis (TSC) prenatally. At the authors' center, all children with TSC undergo a noninvasive cardiac investigation. The required medical and/or cardiosurgical management and outcomes were noted for all the children in the present study.

Table 1. Characteristics of the study group of 30 children with cardiac tumors

Study group, n	30
Female/male, n (%)	17 (57%)/13 (43%)
Age at diagnosis, median (range)	44 days, (1 day to 16 years)
Family history of TSC, n (%)	3 (10%)
Prenatal diagnosis, n (%)	3 (10%)
Cardiac symptoms at the time of diagnosis, n (%)	25 (83%)
Rhabdomyoma diagnosed on the basis of echo exam, n (%)	22 (73%)
Fibroma suspected, n (%)	8 (27%)
CT/MRI, n (%)	8 (27%)
Neurological consultation, n (%)	30 (100%)

n – number of children; TSC – tuberous sclerosis.

Results

The baseline characteristics of the study group are presented in Table 1. There were 30 children, 17 females (57%) and 13 males (43%). Their ages ranged from 1 day to 16 years (median: 44 days) at the time of diagnosis.

Table 2 shows the patients' clinical presentation at the time the cardiac tumors were diagnosed. A positive family history of TSC was present in 3 cases (10%), and in all of them the diagnosis of TSC was made prenatally and confirmed postnatally.

Table 2. Clinical symptoms and signs at diagnosis

Study group, n	30
Family history of TSC, n (%)	3 (10%)
Heart failure, n (%)	3 (10%)
Heart murmur, n (%)	15 (50%)
Arrhythmias and conduction abnormalities, n (%)	7 (23%)
No cardiac symptoms, n (%)	5 (17%)

n – number of children; TSC – tuberous sclerosis.

Five neonates (17%) were diagnosed immediately postpartum – 3 were referred due to the presence of a heart murmur and 2 because of an irregular heart rate.

In 3 other cases (10%), respiratory distress and heart failure with low cardiac output were the reasons for the cardiac consultation.

Other signs of cardiac tumors in the study group were heart murmurs in 15 cases (50%) and various forms of arrhythmia in 7 cases (23%). There were 5 children (17%) who did not manifest any cardiac symptoms. They had already undergone a cardiac investigation due to a clinical picture of tuberous sclerosis in 3, for a sport eligibility qualification in 1, and because of the prolongation of an upper respiratory tract infection in 1.

Electrocardiograms and 24-h Holter ECG monitoring showed abnormalities and a variety of arrhythmias in 7 children at the time of diagnosis. Nonspecific ST-T changes were present in 3 cases and aberrant intraventricular conduction in 2 cases. Coexisting right and left ventricular hypertrophy and right atrial enlargement were noted in 3 other cases. Tachycardia was discovered in 3 cases and bradycardia in 2 others (in 1 case with a prolonged PR interval). Six children had more than 1 abnormality at the time of diagnosis.

Images

Echocardiography was the primary imaging method used in all patients (Figs. 1–4). Rhabdomyoma (Figs. 1a, 1c, 1e, 1f) was diagnosed in 22 cases; in 16 of them tuberous sclerosis was confirmed during the follow-up period. All 16 patients who had confirmed TSC showed multiple rhabdomyomas on echocardiography. The size of the tumors ranged from 4 mm to 3 cm.

Recently, computed tomography scanning and magnetic resonance imaging have been carried out in selected cases. The indications for scanning were a suspicion of tumor infiltration (MRI in 2 cases – Figs. 2c–d), difficulties in determining the tumor morphology and its boundaries by echocardiography (MRI in 3 cases and CT in 3 others). In the remaining 8 cases, fibroma was the most likely diagnosis based on echo and MRI examinations (Figs. 2a–d, 3a–b).

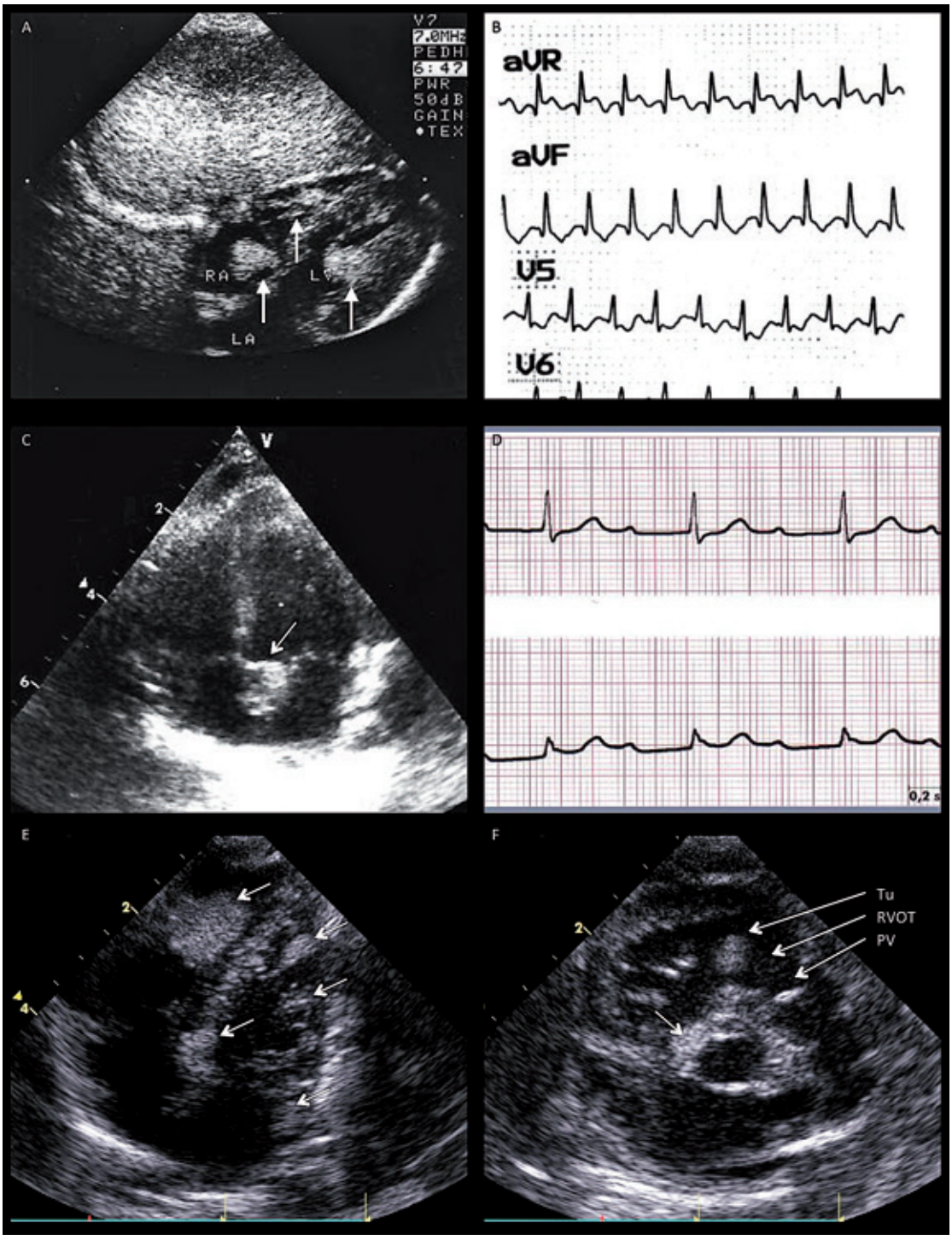

Fig. 1. Main features of cardiac tumors in children

Fig. 1a. Cardiac rhabdomyomas in an infant

Fig. 1b. Surface electrocardiogram, showing supraventricle tachycardia. Further diagnostic procedures revealed Wolff-Parkinson-White syndrome. Transthoracic echocardiography in a modified 4-chamber view revealed round echogenic masses (arrows) bulging into the cavity of the left and right ventricles. The infant was found to have tuberous sclerosis

Fig. 1c–d. Transthoracic echocardiography in a 4-chamber view (c) in a 2-month-old girl, performed because of first degree atrioventricular block (d). A pathological mass is seen in the left atrium (arrow)

Fig. 1e–f. Cardiac rhabdomyomas. Neonatal echocardiography in a 4-chamber view (e) and in a short-axis view (f), showing multiple homogenous left and right ventricular cavity masses (arrows)

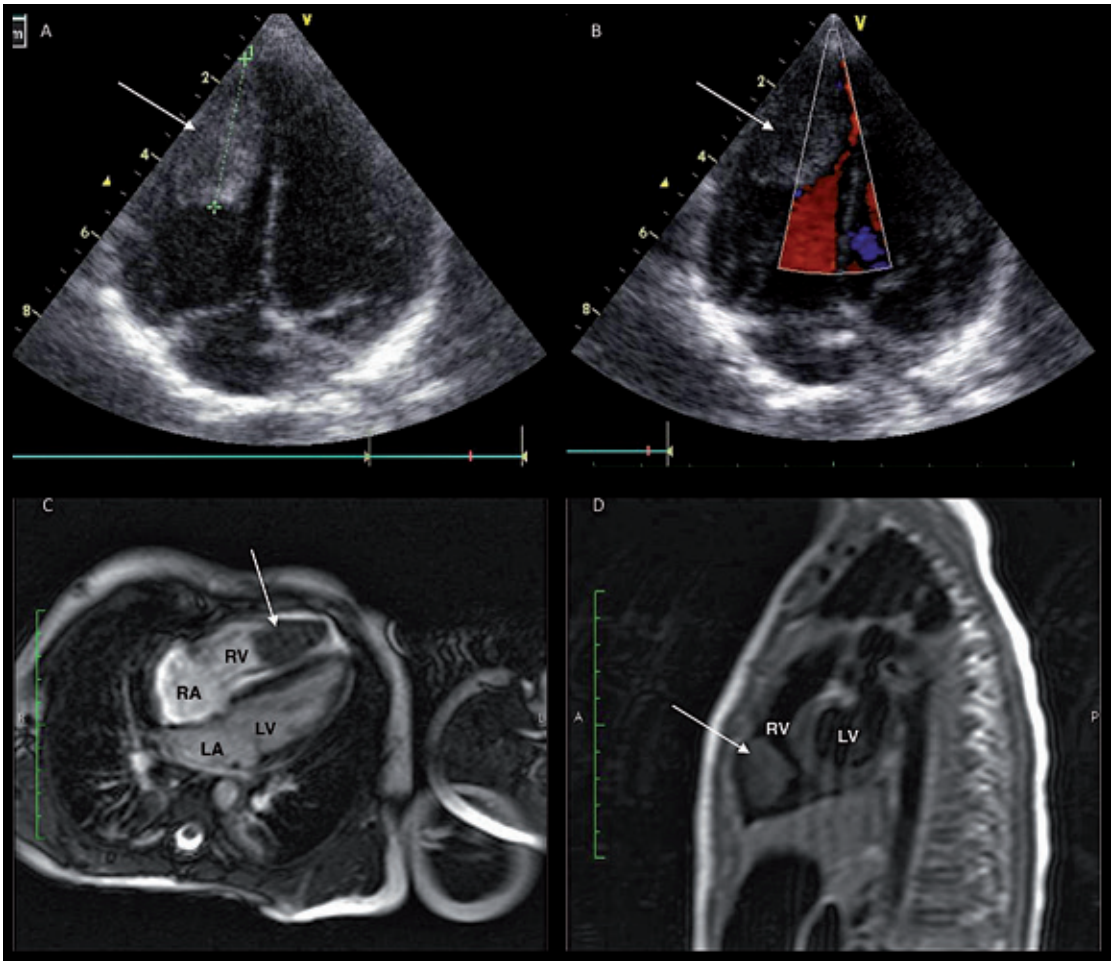

Fig. 2a–b. Transthoracic echocardiography of a child at the age of 2 years showing a right ventricular tumor

Fig. 2c–d. Cardiac magnetic resonance image showing a fibroma in the right ventricle

Management and outcomes

Table 3 presents the treatment regimens and outcomes in the study group.

Three infants (10%) were severely hemodynamically compromised and required cardiac surgery. In 2 of them the left ventricle outflow tract was obstructed by the tumor, and in the third both left and right outflow tracts were obstructed by multiple rhabdomyomas. In these 3 cases subtotal tumor excision was performed. This enabled histological evaluations, which confirmed the presence of rhabdomyomas.

Table 3. Treatment regimens and outcomes

Study group, n	30
No medical/cardiosurgical management, n (%)	22 (73%)
Surgery < age 1 year, n (%)	3 (10%)
Surgery > age 1 year, n (%)	0 (0%)
Reoperation, n (%)	0 (0%)
Number of children treated with antiarrhythmic drugs, n (%)	4 (13%)
Spontaneuos resolution of the tumor, n (%)	12 (40%)
Death, n (%)	1 (3%)

n – number of children.

Three neonates and 1 other infant with various forms of arrhythmias (due to supraventricular re-entry tachycardia in 3 of them and focal atrial tachycardia in 1 case) were successfully treated for their symptoms with several antiarrhythmic drugs, applied according to standard guidelines.

Overall rhythm abnormalities were noted in 10 patients during the follow-up period: 2 had premature ventricular beats (Figs. 3c–f), 3 had supraventricular premature beats, 1 had ectopic atrial tachycardia, 3 had supraventricular re-entry tachycardia (Fig. 1b) and 1 had first degree atrioventricular block (Fig. 1d). The rest of the children had no documented arrhythmias.

Follow-up results

Table 4 presents the follow-up results. As of August 2015, 29 of the patients (97%) were alive. There was 1 non-cardiac death: A patient with TSC and cardiac rhabdomyomas without the need for surgical intervention who died suddenly at home at the age of 7 years. The child was severely mentally handicapped; at the parents' request, no autopsy was conducted.

At the last follow-up visit, 4 of the children were in functional class I according to the Ross classification

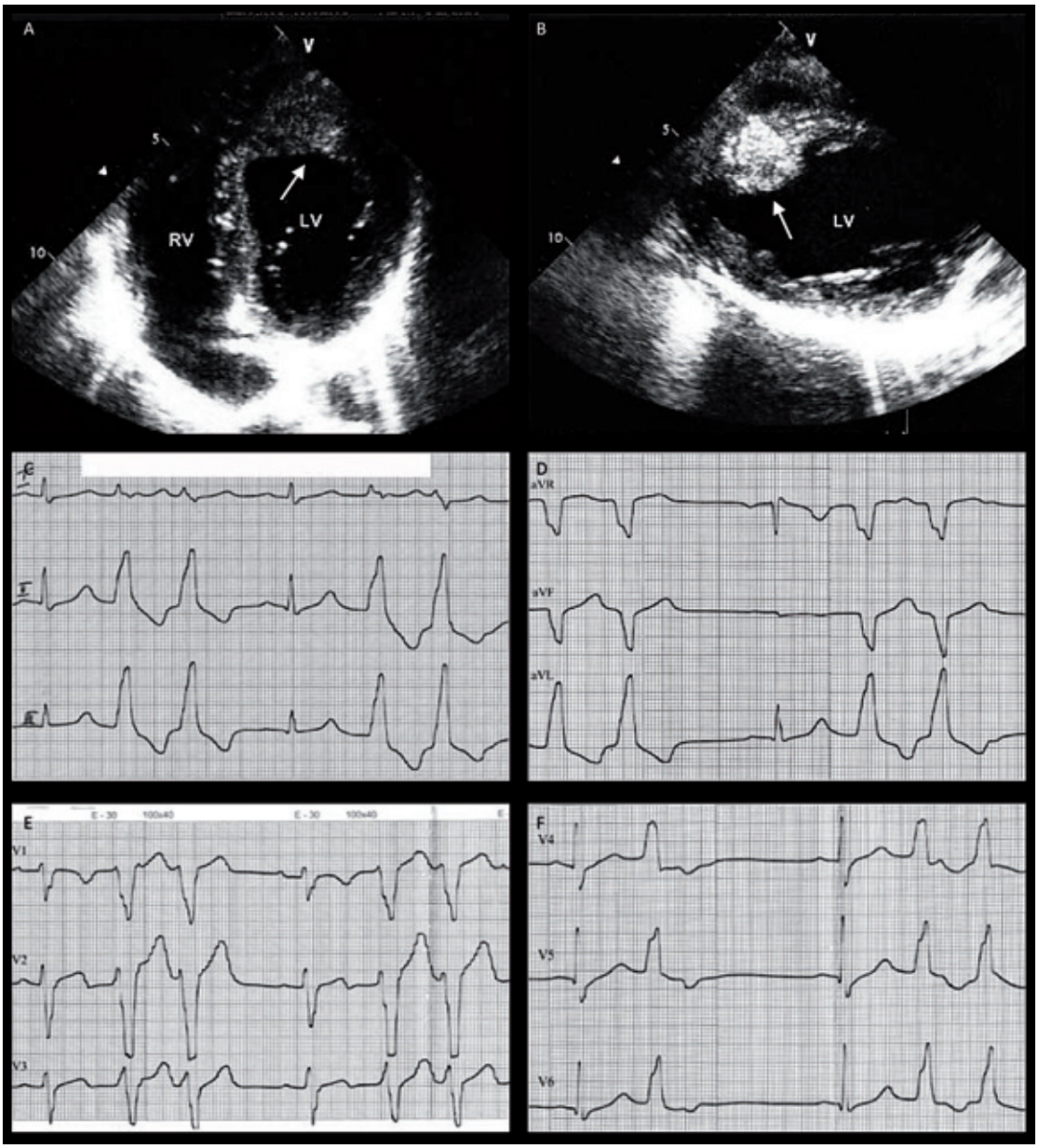

Fig. 3a–b. Transthoracic echocardiography in a modified 4-chamber view and in the parasternal long-axis view, performed in a teenage athlete due to ventricular ectopies revealed in surface electrocardiogram (3c–f). A pathological mass is visible in the apex of the left ventricle (arrow). Cardiac magnetic resonance image confirmed a fibroma in the left ventricle

Table 4. Follow-up data at last cardiac evaluation

Alive, n (%)	29 (97%)
NYHA I	25/25
Ross (or modified Ross) I	4/4
Arrhythmias and conduction disturbances, n (%) VPB SVPB AET AVRT AVB I	 2 (7%) 3 (10%) 1 (3%) 3 (10%) 1 (3%)
TSC confirmation and neurological follow-up, n (%)	16 (55%)

VPB – ventricular premature beat; SVPB – supraventricular premature beat; AET – atrial ectopic tachycardia; AVRT – atrioventricular reentry tachycardia; AVB I – first degree atrioventricular block.

(or modification thereof), and 25 were in the NYHA functional class I.

Spontaneous resolution of the cardiac tumor occurred in 12 of the 16 children with a confirmed diagnosis of tuberous sclerosis. The mean time for the resolution of rhabdomyomas was 4 years, based on serial echo examinations (Fig. 4). In the rest of the study group, there was no progression on echo or MRI visualization, and no further indication for cardiac surgery. During observation periods of up to 20 years, none of the patients required a re-operation. Cardiac pharmacotherapy was not required by 22 children in the study group (73%).

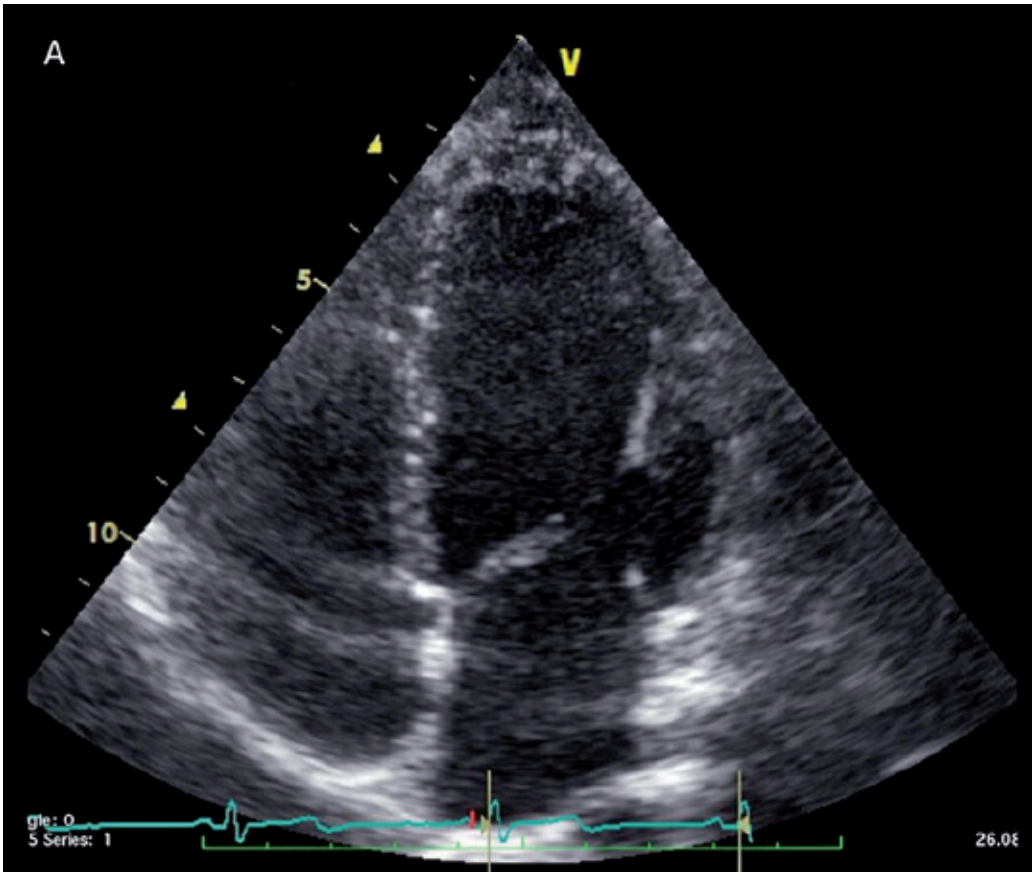

Fig. 4. Transthoracic echocardiography (4-chamber view) of a child with a confirmed diagnosis of tuberous sclerosis. Regression of rhabdomyomas after 4 years of follow-up

Discussion

Tumor incidence

Primary cardiac tumors are found in approximately 1 in 10 000 (0.01%) of routine post-mortem examinations of patients of all ages.[4–7] Simcha et al. found an incidence of 8/10 000 (0.08%) in infants and children.[8] Between 1950–1970 they diagnosed primary cardiac tumors in 8 children based on data from clinical symptoms, electrocardiography, angiocardiography and in only 2 cases were tumors found during the autopsy. Although cardiac tumors are uncommon, the number of cardiac lesions detected annually has increased significantly during recent decades, due to improved imaging techniques.[9] During the 20 years from 1995 to 2015 the present authors diagnosed 30 cases of cardiac tumors in children at the Medical University of Gdańsk Department of Pediatric Cardiology and Congenital Heart Defects.

Clinical presentation and required management

In the present study group, nonspecific heart murmurs (in 15 out of 30 children) and arrhythmias (in 7 out of 30 children) were the most frequent symptoms leading to cardiac consultations. This coincides with other authors' observations.[1,2,10,11] However, it should be emphasized that after birth, patients may not present obvious clinical findings, despite an extensive cardiac involvement.

In cases of newborns and infants with large rhabdomyomas who are critically symptomatic, presenting respiratory distress and congestive heart failure, cardiac surgery is required.[12–14] In the present study group 3 infants (10%) required cardiac surgery due to severe hemodynamic disturbances. Thomas-de-Montprevillea et al. presented almost 20 years of surgical experience with 59 patients (both adults and children), and one of their conclusions was that "there is a group of heart tumors requiring surgery mainly because of the mass effect".[15] Burke and Virmani reviewed the literature concerning heart tumors in children, and with regards to rhabdomyomas they concluded: "Due to a natural history of spontaneous regression of rhabdomyomas many patients are followed without surgery. However, serious symptoms may precipitate the need for surgical resection".[10]

As Moradian et al. wrote: "Sudden death has been attributed to arrhythmias in pediatric patients of all ages. These arrhythmias may be a result of either severe hemodynamic compromise or contiguous location of tumors to the conduction system"[16]; another reported cause is ectopic tachyarrhythmia caused by a tumor.[12] In the literature, all common rhythm disturbances have been reported.[10,13,16–19] In a review of 224 fetuses and neonates with cardiac tumors collected from the literature, Isaacs[12]

confirmed the observations of Moradian et al.[16] and his conclusions were similar to the present authors'.

While it is well established that cardiac rhabdomyomas are often the only manifestation of TSC[2,12,13,20], it is difficult to estimate the true incidence of rhabdomyomas, as this tumor often regresses over time. The published data suggest that in 30% to 91% of cases, cardiac rhabdomyomas are associated with TSC.[2,21] In a study by Beghetti et al. 41% of cardiac rhabdomyomas cases were associated with TSC[2], in contrast to the data reported by Holley et al., who stated that the incidence was 91%.[21] Despite the considerable discrepancies in the frequency of coexisting rhabdomyiomas and TSC, both Beghetti et al. and Holley et al. emphasize that the seizures were a leading symptom of TSC. In tuberous sclerosis it is not the cardiac manifestations but rather the cerebral ones that are crucial to the prognosis.[13,18] In the present study all the children with cardiac tumors underwent neurological consultations, and all the children with TSC have been under regular neurological supervision.

Diagnosis

Lately the diagnosis for most patients with cardiac tumors is established before they are one year old, or even prenatally.[12,18] Verhaaren et al. reported 87 cases with a prenatal diagnosis of rhabdomyoma.[22]

As Moradian et al. wrote: "Fetal cardiac rhabdomyomas, albeit rare, are the most common fetal cardiac tumors. More than 60% of prenatally detected cardiac tumors are rhabdomyomas, and these are often associated with tuberous sclerosis".[16]

In most cases it is not easy to obtain a cardiac tumor biopsy, either by percutaneous or catheter biopsy; therefore, imaging plays a central role in the evaluation of cardiac tumors.[3,23] The primary imaging modality is echocardiography, but in doubtful cases other imaging modalities are employed to enhance visualization. Other centers confirm that MRI is an important tool in the evaluation of cardiac neoplasms, helping to accurately predict the type of cardiac tumor and establish a treatment strategy.[3,24,25,26]

Data from the literature and the results of the present study show that most primary cardiac tumors that occur in children and young people are benign neoplasms.[1,2,27,28,29,30] According to previous research, rhabdomyomas and fibromas are the most frequently occurring cardiac tumors in children.[1,2] The present authors' long-term observations support this statement.

The clinical presentation of cardiac tumors, along with morphologic findings obtained from echocardiography with additional CT and MRI in selected cases, now gives enough information for the identification of different tumor types. The scope and need for future multicenter research in this field should be considered.

Spontaneous tumor regression

Beghetti et al.[2] reported partial or complete spontaneous regression in 54% of 44 analyzed patients. In the present study, spontaneous resolution of the tumor occurred in 12 out of 16 children with a confirmed diagnosis of tuberous sclerosis (75%) in a mean time of 4 years, based on repeated echo examinations. In the rest of the study group there was no progression on echo and/or MRI visualization, and no further indication for a cardiac surgery.

Conclusions

The symptomatology of cardiac tumors in children can vary greatly: from the absence of any symptoms to a heart failure and respiratory distress requiring surgical intervention. Due to the individual course of the disease, all patients, even asymptomatic ones, require regular cardiac follow-ups, including imaging and electrocardiographic procedures. Currently, the diagnosis of cardiac tumors relies almost exclusively on noninvasive imaging techniques: echocardiography, CT and/or MRI. The authors' observations confirm the fact that the most common cardiac tumor in children is rhabdomyoma, which may disappear spontaneously in the majority of patients over the long term. Most patients with cardiac tumors do not require treatment.

Limitations

As a retrospective study, the data collection was limited because of the long follow-up period. The available medical records were limited to those obtained as part of routine cardiac care at the authors' center.

References

1. Becker AE. Primary heart tumors in the pediatric age group: A review of salient pathologic features relevant for clinicians. *Pediatr Cardiol.* 2000;21:317–323.
2. Beghetti M, Gow RM, Haney I, Mawson J, Williams WG, Freedom RM. Pediatric primary benign cardiac tumors: A 15-year review. *Am Heart J.* 1997;134:1107–1114.
3. Hoey ETD, Shahid M, Ganeshan A, Baijal S, Simpson H, Watkin RW. MRI assessment of cardiac tumours: Part 1, multiparametric imaging protocols and spectrum of appearances of histologiacally benign lesions. *Quant Imaging Med Surg.* 2014;4:478–488.
4. Reynen K. Frequency of primary tumors of the heart. *Am J Cardiol.* 1996;77:107–116.
5. Uzun O, Wilson DG, Vujanic GM, Parsons JM, De Giovanni JV. Cardiac tumours in children. *Orphanet J Rare Dis.* 2007;2:11–25.
6. Gunter T, Schreiber Ch, Noebaur Ch, Eicken A, Lange R. Treatment strategies for pediatric patients with primary cardiac and pericardial tumors: A 30-year review. *Pediatr Cardiol.* 2008;29:1071–1076.
7. Marx GR, Moran AM. Cardiac tumors. In: Allen HD, Driscoll DJ, Shaddy RE, Feltes TF, eds. *Heart Disease in infants, children and adolescents including the fetus and young adults.* 8th ed. Moss and Adams', Lippincott Williams & Wilkins;2013:1549–1564.
8. Simcha A, Wells BG, Tynan MJ, Waterston DJ. Primary cardiac tumours in childhood. *Arch Dis Child.* 1971;46:508–514.

9. Gusman M, Servaes S, Feygin T, Degenhardt K, Epelman M. Multimodal imaging in the prenatal diagnosis of tuberous sclerosis complex. *Case Rep Pediatr.* 2012;925–646.
10. Burke A, Virmani R. Pediatric heart tumors. *Cardiovascular Pathology.* 2008;17:193–198.
11. Freedom RM, Lee KJ, MacDonald C, Taylor G. Selected aspects of cardiac tumors in infancy and childhood. *Pediatr Cardiol.* 2000;21: 299–316.
12. Isaacs H. Fetal and neonatal cardiac tumors. *Pediatr Cardiol.* 2004; 25:252–273.
13. Kwiatkowska J, Aleszewicz-Baranowska J, Komorowska W, Siebert J. Rodzinne występowanie choroby Bourneville'a-Pringle'a: opis przypadku. *Fam Med Prim Care Rev.* 2008;10:1212–1214.
14. Perdeus P, Werner B, Kaliciński Z. Cardiac tamponade in a neonate with cardiac tumour. *Kardiol Pol.* 2006;64:309–311.
15. Thomas-de-Montprevillea V, Nottinb R, Dulmeta E, Serrafc A. Heart tumors in children and adults: clinicopathological study of 59 patients from a surgical center. *Cardiovascular Pathology.* 2007; 16:22– 28.
16. Moradian M, Dalili M, Merajie M. Prenatal diagnosis of tuberous sclerosis by fetal echocardiography with an unusual clinical course after birth. *Arch Cardiovasc Image.* 2013;1:83–85.
17. Sallee D, Spector ML, van Heeckeren DW, Patel CR. Primary pediatric cardiac tumors: A 17-year experience. *Cardiol Young.* 1999;9:155–162.
18. Aleszewicz-Baranowska J, Potaż P, Kwiatkowska J, Ereciński J, Komorowska-Szczepańska W. Komorowe zaburzenia rytmu u młodego sportowca z guzem serca: koincydencja czy przyczyna? *Fam Med Prim Care Rev.* 2009;11:812–814.
19. Bang K, Kim YH, Kim CS, Lee SL, Kwon TC. Supraventricular tachycardia in neonate with cardiac rhabdomyoma and tuberous sclerosis. *Korean J Pediatr.* 2008; 51:766–770.
20. Bosi G, Lintermans JP, Pellegrino PA, Svaluto-Moreolo G, Vliers A. The natural history of cardiac rhabdomyoma with and without tuberous sclerosis. *Acta Paediatr.* 1996;85:928–931.
21. Holley DG, Martin GR, Brenner JI, et al. Diagnosis and management of fetal cardiac tumors: A multicenter experience and review of published reports. *J Am Coll Cardiol.* 1995;26:516–20.
22. Verhaaren HA, Vanakker O, De Wolf D, Suys B, Francois K, Matthys D. Left ventricular outflow obstruction in rhabdomyoma of infancy: Meta-analysis of the literature. *J Pediatr.* 2003;143:258–263.
23. Kaminaga T, Takeshita T, Kimura I. Role of magnetic resonance imaging for evaluation of tumors in the cardiac region. *Eur Radiol.* 2003;13:L1–L10.
24. Beroukhim RS, Prakash A, Buechel ERV, et al. Characterization of cardiac tumors in children by cardiovascular magnetic resonanse imaging. *JACC.* 2011;10:1044–1054.
25. Sparrow PJ, Kurian JB, Jones TR, Sivananthan MU. MR Imaging of Cardiac Tumors. *Radio Graphics.* 2005;25:1255–1276.
26. Kiaffas MG, Powell AJ, Geva T. Magnetic resonance imaging evaluation of cardiac tumor characteristics in infants and children. *Am J Cardiol.* 2002; 89: 1229–1233.
27. Coard KCM. Primary tumors of the heart: Experience at the University Hospital of the West Indies. *Cardiovascular Pathology.* 2007;16: 98–103.
28. Yu K, Liu Y, Wang H, Hu S, Long C. Epidemiological and pathological characteristics of cardiac tumors: A clinical study of 242 cases. *Interact Cardio Vasc Thorac Surg.* 2007;6:636–639.
29. ElBardissi AV, Dearani JA, Daly RC, et al. Survival after resection of primary cardiac tumors. A 48-year experience. *Circulation.* 2008; 118(Suppl 1):S7–S15.
30. Kamiya H, Yasuda T, Nagamine H, et al. Surgical treatment of primary cardiac tumors 28 years' experience in Kanazawa University Hospital. *Jpn Circ J.* 2001;65:315–319.

Serum leptin levels and GHR-d3/fl gene polymorphism in acromegalic patients with thyroid nodules

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Abstract

Background. Acromegaly is a rare and serious syndrome that is commonly associated with pituitary neoplasms. Thyroid multinodular disease is a common finding in acromegaly. Leptin is a polypeptide hormone, and studies have shown that it can increase cell proliferation and inhibit apoptosis.

Objectives. The aim of the study was to determine the relationship of serum leptin levels with certain blood parameters and determine if growth hormone receptor (GHR)-d3/fl gene polymorphism is associated with thyroid nodules in acromegalic patients.

Material and methods. A total of 24 acromegalic patients with or without thyroid nodules were included in the study. Gene polymorphisms and blood parameters were examined.

Results. A marked increase was observed in serum leptin concentration in acromegalic patients with thyroid nodules compared to patients without them ($p < 0.05$). GH levels were lower in patients without nodules than in patients with nodules ($p < 0.05$). Blood glucose levels were higher in patients with nodules compared to those without them ($p < 0.05$), and the presence of thyroid nodules was associated with decreased blood low-density lipoprotein (LDL) levels compared to patients without nodules ($p < 0.05$). A significant relationship was observed between growth hormone receptor (GHR)-d3/fl gene polymorphism and leptin levels in acromegalic patients with thyroid nodules ($p < 0.001$).

Conclusions. These data from acromegalic patients indicate that thyroid nodules are associated with increased serum leptin, GH and blood glucose levels and with decreased LDL levels. GHR-d3/fl gene polymorphism status was strongly related to higher leptin levels.

Key words: thyroid, gene polymorphism, acromegaly, hormone, endocrinology

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Acromegaly is associated with excessive secretion of growth hormone (GH) and insulin-like growth factor-1 (IGF-1), resulting in bone overgrowth and an increase in soft tissue.[1] Thyroid nodules are a common clinical problem in females. Epidemiological studies have shown the prevalence of palpable thyroid nodules to be approximately 5% in women and 1% in men living in iodine-sufficient parts of the world.[2,3] In contrast, high-resolution ultrasound can detect thyroid nodules in 19–67% of randomly selected individuals, with higher frequencies in women and the elderly.[4] Thyroid enlargement can also be seen in acromegalic patients.[5,6] Long-term elevation of serum GH levels in acromegaly is associated with goiter development and is probably caused by the mitogenic effects of IGF-1.[7]

Changes in enzyme activity may lead to somewhat higher serum triiodothyronine (T3) and lower reverse T3 (rT3) concentrations in patients with active acromegaly, although the levels are usually within physiological ranges.[8,9] The prevalence rates of certain benign and malignant neoplasms are higher in acromegalic patients compared with the healthy population.[10]

Leptin is a potent anorexigenic hormone found in the anterior pituitary and secreted by adipose tissue in proportion to adipose content. It is a product of the obesity (Ob) gene that regulates both satiety and energy expenditure.[11] Leptin is secreted with diurnal fluctuations, and greater secretion is observed in subjects who are obese or who have insulin resistance.[12,13] While a negative influence of thyroid hormones on serum leptin concentrations has been described, some authors report no influence of thyroid hormones on leptin levels.[14–17]

There are many reports concerning the stimulatory effect of leptin on cell mitosis and its involvement in the carcinogenesis of breast, colon, prostate, lung, kidney and ovary cells. Studies have shown that leptin's abilities to increase cell proliferation and inhibit apoptosis are involved in creating certain types of tumors.[18–24] Specifically, increased expression of leptin and its receptor are well documented in papillary thyroid cancer. Similar to other tumor types, some authors reported that leptin exerts oncogenic effects on papillary thyroid carcinoma cells by stimulating cell proliferation and inhibiting apoptosis.[25–27]

The principal regulator of GH sensitivity is the GH receptor (GHR) and consists of an extracellular domain of 246 amino acids, a single transmembrane domain, and a cytoplasmic domain. The encoding gene has 9 exons, but there are 2 isoforms of the GHR in humans generated by the deletion of exon 3 of the gene, resulting in 3 genotypes: homozygous GHR-fl, homozygous GHR-d3, and heterozygous GHR-d3/fl.[28]

Objectives

Acromegaly is frequently associated with thyroid enlargement, and the size is dependent on the duration of GH excess.[5,6] Thyroid multinodular disease is a common finding in acromegaly.[5,7,29,30] The reported frequencies of diffuse goiter and nodular goiter in acromegalic patients are 78–92 and 63%, respectively.[5] Leptin, an Ob gene product, is secreted exclusively by adipocytes and regulates energy balance. Because GH modulates fat mass, it is possible that GH and chronic GH excess affects leptin. The aim of this study was to examine leptin levels in acromegalic patients, with or without thyroid nodules, and to identify any relationship with GHR gene polymorphism.

Material and methods

Study design

The study was performed according to the Helsinki Declaration and was approved by the Ethical Committee of Pamukkale University (09/198). All the patients and volunteers provided written acknowledgement of informed consent for participation. The study group included 24 patients with acromegaly (13 males, 11 females; mean age, 52.04 ± standard deviation [SD] 9.46; range, 30–68 years), including 17 patients with thyroid nodules (9 males and 8 females; mean age, 51.05 ± SD 9.66; range, 19–84 years) and 7 patients without thyroid nodules (4 males, 3 females; mean age, 46 ± SD 17.6; range, 30–68 years). The participants' anthropometric measurements and blood parameters were evaluated.

The diagnosis of acromegaly was established on the basis of criteria proposed by Freda and confirmed by pathological examination of surgically resected tissues[31]. Clinical signs, magnetic resonance imaging (MRI), and GH and IGF-1 levels were assessed to support the diagnosis. Active acromegaly was associated with increased serum levels of GH and IGF-1, and modified sellar morphology visible on MRI, indicating the presence of a pituitary adenoma.

The main inclusion criteria were age over 18 years and the presence of acromegaly.

The patients included in the study were not surgically or medically treated for acromegaly (e.g. with somatostatin, dopamine analogs or GH receptor antagonists) at the time of inclusion in the study. Exclusion criteria included pregnancy, the use of any drugs at the time of the study or current surgical treatment for thyroid nodules. On this basis 11 patients were excluded from the study.

Clinical and laboratory assessment

Height and weight were measured in light clothing without shoes. Body height and weight were measured by a statometer and digital electronic scale, respectively. Body mass index (BMI) was calculated as the patient's weight in kilograms divided by the square of his/her height in meters. The average value of systolic and dia-

stolic blood pressure measurements were taken with the subject in a sitting position at 2- to 3-min intervals after resting for at least 15 min.

Serum levels of glucose, total cholesterol, triglyceride, high- and low-density lipoprotein (HDL and LDL) cholesterol, alkaline phosphatase (ALP), calcium and phosphorus were analyzed with commercial kits (Beckman-Coulter Inc., Brea, USA) in an LX-20 autoanalyzer (Beckman-Coulter Inc., Brea, USA). Levels of serum insulin, free T3, free thyroxine (T4), thyroid-stimulating hormone, prolactin, GH, leptin, IGF-1 and insulin-like growth factor-binding protein 3 (IGFB3) were measured in an Immulite 2000 immunoassay analyzer (Siemens Healthcare, Erlangen, Germany) using the chemiluminescence method.

Genetic analysis

DNA was isolated from peripheral blood with a standard phenol/chloroform extraction method. Genotyping for the d3 gene was performed by the polymerase chain reaction (PCR) method using a personal thermal cycler (Techgene, USA). PCR was conducted in 50 μL reaction mixture containing about 1 μg of DNA sample, 5 μL reaction buffer (x10) include 160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8), 0.1% Tween-20, 5 μL dNTP (2 mM), 3 μL $MgCl_2$ (25 mM), 1 U platinum Taq-polymerase and 100 pmol of each primer. The following primers were used: G1 5'-TGT GCT GGT CTG TTG GTC TG-3', G2 5'-AGT CGT TCC TGG GAC AGA GA-3' and G3 5'-CCT GGA TTA ACA CTT TGC AGA CTC-3' (32). Amplification was performed for 39 cycles comprised of denaturation, extension and annealing at temperatures of 94°C for 30 s, 57°C for 30 s, and 68°C for 30 s, respectively. The final extension time was carried out at 68°C for 10 min. The initial denaturation stage was carried out at 95°C for 2 min. The fragments obtained were electrophoresed in a 1% agarose gel and visualized by ethidium bromide staining under ultraviolet (UV) light. The polymorphism detected by PCR was evident as a 935-bp fragment in the presence of the full-length (fl/fl) fragment and as a 592-bp product in the presence of the deletion (d3/d3). Each sample was described as d3/d3, d3/fl, or fl/fl.

Statistical analysis

Descriptive statistics, proportions for categorical variables, and means and SDs for continuous variables were used to describe the study groups. Comparisons between acromegalic patients with and without thyroid nodules were made with Student's t-tests. The results were expressed as means ± standard error. Mann-Whitney U tests were performed to analyze between-group differences in continuous variables. Values were considered to be statistically significant when $p < 0.05$. Comparisons of gene polymorphisms and leptin levels were made by one-way analysis of variance (ANOVA). Regression analysis was used to tease out the impact of the genotype. All statistical analyses were carried out using SPSS 10.0 software (SPSS Inc., Chicago, USA).

Results

A total of 24 acromegalic patients were included: 13 males (9 with nodules and 4 without) and 11 females (8 with nodules and 3 without). The mean ages of the participants with and without nodules were 51.05 ± 9.66 and 54.57 ± 9.18, respectively ($p > 0.05$). The participants' anthropometric measurements and blood parameters are shown in Table 1. In the patients with nodules, statistically significant increases were observed in IGFB3, blood glucose and leptin levels, and decreases were noted for GH, BMI, homeostasis model assessment (HOMA), LDL and phosphorus (P) levels.

In this study, the frequencies of the GHR-fl/fl, d3/d3, and d3/fl genotypes were 62.5, 29.1, and 8.34%, respectively. There was a strong relationship between gene polymorphism status and leptin levels in acromegalic patients (Table 2). A marked increase was observed in serum leptin levels in patients with the GHR-d3/fl genotype ($p < 0.01$).

Discussion

Acromegaly is an acquired disorder related to excessive production of GH and IGF-1 and characterized by progressive somatic disfigurement and systemic manifestations.[33] Leptin, a neuroregulatory peptide, is secreted by adipose tissue in proportion to adipose content. Because GH modulates fat mass, the study was undertaken to investigate the possible effects of acromegaly on leptin in patients with and without thyroid nodules.

The growth-promoting effects of GH on thyroid follicular cells or a concurrent TSH excess are the conventional hypotheses linking acromegaly to thyrotoxicosis.[30] Current evidence favors a TSH-independent mechanism in most cases.[34] In addition, G protein abnormalities can constitutively activate GH-releasing hormone (GHRH) receptors leading to acromegaly, as well as cause constitutive TSH receptor activation leading to thyrotoxicosis.[35] This may also be a possible mechanism underlying the combination of acromegaly and thyroid nodules studied here.

In this study, serum leptin levels in acromegalic patients with different GHR genotypes (fl/fl, d3/d3, or d3/fl) were examined, and a statistically significant relationship between gene polymorphism status and nodule formation was observed. While no differences were found in homozygous fl and d3 patients, a marked predisposition for thyroid nodule development was observed in patients that were heterozygous (d3/fl). Specifically, patients with the GHR-d3/fl genotype exhibited both increased leptin

Table 1. Mann Whitney U test results of clinical and laboratory characteristics of acromegalic patients with or without thyroid nodule

Parameters	Acromegalic patient with thyroid nodule (n = 17)		Acromegalic patient without thyroid nodule (n = 7)		U	p-value
	mean rank	sum of ranks	mean rank	sum of ranks		
Age (years)	14.07	201.50	11.85	98.50	48.500	ns
BMI (kg/m^2)	14.00	238.00	8.86	62.00	34.000	< 0.05*
Weight (kg)	13.38	227.50	10.36	72.50	44.500	ns
Height (cm)	12.29	209.00	13.00	91.00	56.000	ns
Fasting glucose (mg/dL)	14.41	245.00	7.86	55.00	27.000	< 0.05*
Insulin (µIU/mL)	13.53	203.00	10.00	70.00	42.000	ns
HOMA	13.97	237.50	8.93	62.50	34.500	< 0.05*
GH (ng/L)	11.97	203.50	15.79	96.50	50.500	< 0.05*
IGF1 (ng/L)	12.76	217.00	11.86	83.00	55.000	ns
IGFB3 (µg/L)	11.76	200.00	14.29	100.00	47.000	< 0.05*
Total cholesterol (mg/dL)	13.41	228.00	10.29	72.00	44.000	ns
Triglyceride (mg/dL)	13.35	227.00	10.43	73.00	45.000	ns
LDL (mg/dL)	12.12	206.00	13.43	94.00	53.000	ns
HDL (mg/dL)	12.26	208.50	13.07	91.50	55.000	ns
TSH (µIU/mL)	12.94	220.00	11.43	80.00	52.000	ns
Leptin (ng/mL)	13.24	225.00	9.71	75.00	47.000	< 0.05*
FT3 (pg/mL)	12.15	206.50	13.36	93.50	53.500	ns
FT4 (ng/dL)	11.97	203.50	13.79	96.50	50.500	ns
COR (µg/dL)	13.26	225.50	10.64	74.50	46.000	ns
PRL (ng/mL)	12.88	219.00	11.57	81.00	53.000	ns
CA (mg/dL)	12.29	209.00	13.00	91.00	56.000	ns
P (mg/dL)	13.85	235.50	9.21	64.50	36.500	< 0.05*
ALP (IU/L)	13.16	210.50	9.36	65.50	37.500	ns
Right thyroid lobe (mm)	12.38	210.50	12.79	89.50	57.500	ns
Left thyroid lobe (mm)	13.21	224.50	10.79	75.50	47.500	ns
Isthmus (mm)	10.22	163.50	14.92	89.50	27.000	ns

ns – non-significant, * significant differences between the groups ($p < 0.05$).

Table 2. GHR gene polymorphisms; leptin, IGF-1, and GH levels; and BMI in acromegalic patients with or without thyroid nodule

Patients	n	Leptin level	BMI	IGF-1	GH
fl/fl genotypes with nodule	11	6.44 ± 9.09^a	28.70 ± 4.61	740.54 ± 326.89	15.77 ± 11.96
fl/fl genotypes without nodule	4	3.25 ± 4.74^a	25.72 ± 2.99	436.50 ± 300.98	9.86 ± 7.07
d3/d3 genotypes with nodule	4	7.57 ± 8.60^a	28.60 ± 2.65	393.50 ± 272.94	5.36 ± 3.37
d3/d3 genotypes without nodule	3	4.79 ± 2.80^a	26.93 ± 1.66	1045.00 ± 677.62	31.43 ± 25.93
d3/fl genotypes with nodule	2	28.19 ± 3.46^b	35.15 ± 0.63	563.00 ± 216.37	4.96 ± 9.19
p-value		< 0.01**	ns	ns	ns

** The differences between the means of groups carrying different letters in the same column are statistically significant ($p < 0.01$), ns – non-significant.

levels and a predisposition to nodule formation. Notably, nodules were found in all patients with the GHR-d3/fl genotype (Table 2).

GH plays an important role in the regulation of adiposity and modulates fat deposition and accumulation via regulatory molecules in preadipocytes and adipocytes.[36] Some authors have reported that GH excess is associated with decreased leptin levels and decreased fat mass.[37] In contrast, the present study found that increased leptin levels were associated with decreased GH levels in acromegalic patients with thyroid nodules. Lower leptin levels have been reported in acromegalic patients.[1,38] Different treatments for acromegaly lead to increased leptin levels.[39–41] Collectively, the evidence indicates that leptin secretion is reduced in active acromegaly, presumably reflecting reduced body fat stores. Restoration of GH secretion is as-

sociated with an increase in leptin levels, probably due to an increase in fat stores.[1] Leonhardt et al. reported a significant increase in serum leptin levels in a hypothyroid patient compared to hyperthyroid and normal controls.[42] Similar results were reported by Yoshida et al.[43] Leptin is also related to the progression of some tumors, including thyroid cancers.[18–22,24–27]

In the present study, a marked increase in leptin levels was observed in acromegalic patients with thyroid nodules compared to those without. These findings indicate that the thyroid may have a greater effect on leptin levels than the pituitary. The mechanism underlying the link between leptin and LDL levels is not clear. However, some authors have reported that leptin has a pro-oxidant effect. Previous reports have described leptin having a direct effect on endothelial cell generation of reactive oxygen species that have been shown to play a role in LDL oxidation.[44–46] In this study, decreased LDL levels may be related to higher leptin levels in acromegalic patients with thyroid nodules.

GHR polymorphism has been reported in acromegaly, but to the best of the authors' knowledge, this is the first study investigating anthropometric measurements, blood parameters and gene polymorphism in acromegalic patients with or without thyroid nodules.[32] Although the study included only a small group of patients because of the rarity of the combination of the 2 conditions and the unusual inclusion criteria, the results indicate that thyroid nodules are associated with changes in some parameters in acromegalic patients. Practitioners need to take these results into consideration when treating acromegalic patients. At the same time, although the GHR-d3/fl genotype was a predisposing factor in acromegalic patients in this study, there were no differences in IGF-1 levels in these patients. The possible cause of this may be related to other growth factors, and higher leptin levels may contribute to thyroid nodule development. This is a preliminary result, and further studies are needed.

References

1. Katznelson L. Alterations in body composition in acromegaly. *Pituitary*. 2009;12(2):136–142.
2. Tunbridge WMG, Evered DC, Hall R, et al. The spectrum of thyroid disease in a community: The Whickham Survey. *Clin Endocrinol (Oxf)*. 1977;7(6):481–493.
3. Vander JB, Gaston EA, Dawber TR. The significance of nontoxic thyroid nodules. Final report of a 15-year study of the incidence of thyroid malignancy. *Ann Intern Med*. 1968;69(3):537–540.
4. Tan GH, Gharib H. Thyroid incidentalomas: Management approaches to nonpalpable nodules discovered incidentally on thyroid imaging. *Ann Intern Med*. 1997;126(3): 226–231.
5. Gasperi M, Martino E, Manetti L, et al. Acromegaly study group of Italian Society of Endocrinology: Prevalence of thyroid diseases in patients with acromegaly: Results of an Italian multicenter study. *J Endocrinol Invest*. 2002;25(3):240–245.
6. Herrmann BL, Baumann H, Janssen OE, Gorges R, Schmid KW, Mann K. Impact of disease activity on thyroid diseases in patients with acromegaly: Basal evaluation and follow-up. *Exp Clin Endocrinol Diabetes*. 2004;112(5):225–230.
7. Miyakawa M, Saji M, Tsushima T, Wakai K, Shizume K. Thyroid volume and serum thyroglobulin levels in patients with acromegaly: Correlation with plasma insulin-like growth factor 1 levels. *J Clin Endocrinol Metab*. 1988;67(5):973–978.
8. Geelhoed-Duijvestijn PH, Bussemaker JK, Roelfsema F. Changes in basal and stimulated TSH and other parameters of thyroid function in acromegaly after transsphenoidal surgery. *Acta Endocrinol*. 1989;121(2):207–215.
9. Roelfsema F, Frolich M. Pulsatile thyrotropin release and thyroid function in acromegalics before and during subcutaneous octreotide infusion. *J Clin Endocrinol Metab*. 1991;72 (1):77–82.
10. Baldys-Waligorska A, Krzentowska A, Golkowski F, Sokolowsk G, Hubalewska-Dydejczyk A. The prevalence of benign and malignant neoplasms in acromegalic patients. *Endokrynol Pol*. 2010;61(1): 29–34.
11. Rowland NE, Morien A, Li BH. The physiology and brain mechanisms of feeding. *Nutrition*. 1996;12(9):626–639.
12. Licinio J, Negrao AB, Mantzoros C, et al. Sex differences in circulating human leptin pulse amplitude: clinical implications. *J Clin Endocrinol Metab*. 1998;83(11):4140–4147.
13. Lonnqvist F, Wennlund A, Arner P. Relationship between circulating leptin and peripheral fat distribution in obese subjects. *Int J Obes Relat Metab Disord*. 1997;21(4):255–260.
14. EscobarMorreale HF, delRey FE, deEscobar GM. Thyroid hormones influence serum leptin concentrations in the rat. Endocrinology. 1997;138(10);4485–4488.
15. Santini F, Marsili A, Mammoli C, et al. Serum concentrations of adiponectin and leptin in patients with thyroid dysfunctions. *J Clin Endocrinol Invest*. 2004;27(2):RC5–RC7.
16. Sesmilo G, Casamitjana R, Halperin I, Gomis R, Vilardell E. Role of thyroid hormones on serum leptin levels. *Eur J Endocrinol*. 1998;139(4):428–430.
17. Sreenan S, Caro JF, Refetoff S. Thyroid dysfunction is not associated with alterations in serum leptin levels. *Thyroid*. 1997;7(3):407–409.
18. Brauner R, Trivin C, Zerah M, et al. Diencephalic syndrome due to hypothalamic tumour: A model of the relationship between weight and puberty onset. *J Clin Endocrinol Metab,* 2006;91(7):2467–2473.
19. Garofalo C, Surmacz E. Leptin and cancer. *J Cell Physiol*. 2006;207(1): 12–22.
20. Hoda MR, Keely SJ, Bertelsen LS, Junger WG, Dharmasena D, Barrett KE. Leptin acts as a mitogenic and antiapoptotic factor for colonic cancer cells. *Brit J Surg*. 2007;94(3):346–354.
21. Jaffe T, Schwartz B. Leptin promotes motility and invasiveness in human colon cancer cells by activating multiple signal-transduction pathways. *Int J Cancer*. 2008;123(11):2543–2556.
22. Li L, Gao Y, Zhang LL, He DL. Concomitant activation of the JAK/STAT3 and ERK1/2 signaling is involved in leptin-mediated proliferation of renal cell carcinoma Caki-2 cells. *Cancer Biol Therapy*. 2008;7(11):1787–1792.
23. O'Brien SN, Welter BH. Price TM. Presence of leptin in breast cell lines and breast tumors. *Bioch Bioph Res Co*. 1999;259(3):695–698.
24. Ribeiro R, Arauj A, Lopes C, Medeiros R. Immunoinflammatory mechanisms in lung cancer development: Is leptin a mediator? *J Thorac Oncol*. 2007;2(2):105–108.
25. Mantzoros CS, Rosen HN, Greenspan SL, Flier JS, Moses AC. Short-term hyperthyroidism has no effect on leptin levels in man. *J Clin Endocrinol Metab*. 1997;82(2):497–499.
26. Uddin S, Bavi P, Siraj AK, et al. Leptin-R and its association with PI3K/AKT signaling pathway in papillary thyroid carcinoma. *Endocr Relat Cancer*. 2010;17(1):191–202.
27. Valcavi R, Zini M, Peino R, Casanueva FF, Dieguez C. Influence of thyroid status on serum immunoreactive leptin levels. *J Clin Endocrinol Metab*. 1997;82(5):1632–1634.
28. Toyoshiman MT, Castroneves LA, Costalonga EF, et al. Exon 3-deleted genotype of growth hormone receptor (GHRd3) positively influences IGF-1 increase at generation test in children with idiopathic short stature. *Clin Endocrinol*. 2007;67(4):500–504.
29. Cheung NW, Boyages SC. The thyroid gland in acromegaly: An ultrasonographic study. *Clin Endocrinol*. 1997;46(5):545–549.
30. Kasagi K, Shimatsu A, Miyamoto S, Misaki T, Sakahara H, Konishi J. Goiter associated with acromegaly: sonographic and scintigraphic findings of the thyroid gland. *Thyroid*. 1999;9(8):791–796.

31. Freda PU. Current concepts in the biochemical assessment of the patient with acromegaly. *Growth Horm IGF Res*. 2003;13:171–184.
32. Montefusco L, Filopanti M, Ronchi CL, et al. d3-Growth hormone receptor polymorphism in acromegaly: Effects on metabolic phenotype. *Clin Endocrinol.* 2010;72(5):661–667.
33. Holdaway IM, Rajasoorya C. Epidemiology of acromegaly. *Pituitary*. 1999;2(1):29–41.
34. Marzullo P, Cuocolo A, Ferone D, et al. Cardiac effect of thyrotoxicosis in acromegaly. *J Clin Endocrinol Metab*. 2000;85(4):1426–1432.
35. Spada A, Lania A, Ballare E. G protein abnormalities in pituitary adenomas. *Mol Cell Endocrinol.* 1998;25(142):1–14.
36. Bluher S, Kratzsch J, Kiess W. Insulin-like growth factor I, growth hormone and insulin in white adipose tissue. *Best Pract Res Clin Endocrinol Metab*. 2005;19(4):577–587.
37. Damjanovic SS, Petakov MS, Raicevic S, et al. Serum leptin levels in patients with acromegaly before and after correction of hypersomatotropism by trans-sphenoidal surgery. *J Clin Endocrinol Metab.* 2000;85(1):147–154.
38. Bolanowski M, Milewicz A, Bidzinska B, Jedrzejuk D, Daroszewski J, Mikulski E. Serum leptin levels in acromegaly – a significant role for adipose tissue and fasting insulin/glucose ratio. *Med Sci Monit.* 2002;8:CR685–CR689.
39. Paramo C, Fluiters E, de la Fuente J, Andrade A, Garcia-Mayor RV. Monitoring of treatment success in patients with acromegaly: The value of serum insulin-like growth factor binding protein-3 and serum leptin measurements in comparison to plasma insulin-like growth factor I determination. *Metabolism*. 2001;50(9):1117–1121.
40. Parkinson C, Whatmore AJ, Yates AP, et al. The effect of pegvisomant-induced serum IGF-I normalization on serum leptin levels in patients with acromegaly. *Clin Endocrinol (Oxf)*. 2003;59(2):168–174.
41. Tan KC, Tso AW, Lam KS. Effect of Sandostatin LAR on serum leptin levels in patients with acromegaly. *Clin Endocrinol (Oxf).* 2001; 54(1):31–35.
42. Leonhardt U, Ritzel U, Schafer G, Becker W, Ramadori G. Serum leptin levels in hypo- and hyperthyroidism. *J Endocrinol.* 1998;157(1): 75–79.
43. Yoshida T, Momotani N, Hayashi M, Monkawa T, Ito K, Saruta T. Serum leptin concentrations in patients with thyroid disorders. *Clin Endocrinol.* 1998;48(3):299–302.
44. Bouloume A, Marumo T, Lafontan M, Busse R. Leptin induces oxidative stress in human endothelial cells. *FASEB J.* 1999;13(10):1231–1238.
45. Yamagishi SI, Edelstein D, Du XL, Kaneda Y, Guzman M, Brownlee M. Leptin induces mitochondrial superoxide production and monocyte chemoattractant protein-1 expression in aortic endothelial cells by increasing fatty acid oxidation via protein kinase. *J Biol Chem.* 2001;276(27):25096–25100.
46. Holvoet P, Collen D. Oxidized lipoproteins in atherosclerosis and thrombosis. *FASEB J*. 1994;8(15):1279–1284.

Closed reduction and percutaneous annulated screw fixation in the treatment of comminuted proximal humeral fractures

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Abstract

Background. Displaced proximal humeral fractures remain a challenge to orthopedic surgeons.

Objectives. The purpose of this study was to evaluate the functional and radiological outcomes of patients with comminuted proximal humeral fractures treated with closed reduction and percutaneous screw fixation (CRPF).

Material and methods. The authors retrospectively reviewed 38 cases of displaced proximal humeral fractures (2-, 3- or 4-part fractures according to the Neer classification) that were treated using the CRPF technique from May 2009 to April 2013. From this group 26 patients were followed up for a period ranging from 9 to 24 months (averaging 12.9 months) and evaluated for the functional and radiological outcomes by a series of standard questionnaires and measurements.

Results. The fractures in all 26 patients were healed within an average time of 14.6 weeks (ranging from 11 to 27 weeks), and the mean interval between the operation and fully functional activity was 18.6 weeks (ranging from 15 to 32 weeks). At the final follow-up visit, no patient showed shoulder instability; the mean range of abduction motion was 146.5° (ranging from 72° to 180°). For all patients, no statistically significant difference in the functional outcomes was observed between their 6-month and final follow-up visits; or in the radiological findings between their immediate post-operative and final follow-up examinations.

Conclusions. The CRPF technique is a safe and effective therapeutic option for comminuted proximal humeral fractures. Good stability is obtained and aggressive impairment of the soft tissue and periosteum around the fracture is avoided, which allows for an early painless range of motion. The technique promotes bone healing, prevents ischemic osteonecrosis of the head of the humerus and leads to few complications.

Key words: functional outcome, radiological outcome, proximal humeral fracture, fracture fixation, percutaneous technique

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Although comminuted proximal humeral fractures are among the most common fractures in the elderly population, treatment of this injury remains a challenge and is still an issue of debate. The Neer classification is one of the most popular systems for assessing fractures of this kind. No displaced or minimally displaced fractures can be treated conservatively with success. Displaced 2-part, 3-part and 4-part fractures should be treated for reduction and stabilization.

Considerable therapeutic options for these fractures have been described in past years.[1] However, for some elderly osteoporosis patients or more comminuted cases, the stability of percutaneous pinning fixation cannot meet the requirements of fixation and early functional activity. Therefore, compressing inter-fracture fragments is of great importance for restoring post-operative motor function.

Percutaneous annulated screw fixation, first described by Chen et al., was developed specifically for comminuted proximal humeral fractures, and appeared to offer improved fixation intensity of these fractures through the use of multiple annulated compression screws oriented in different directions to maximize the screw compression effects and the resistance to displacement.[2] Although this technique is demanding, it can be very effective for unstable 2-part and 3-part fractures and even some 4-part fractures in patients whose bone quality is not good.

The aim of this study was to evaluate the post-operative outcomes of 26 patients with displaced proximal humeral fractures treated with closed reduction and percutaneous screw fixation (CRPF). The authors reviewed the functional outcomes, radiographic outcomes and complications to investigate the hypothesis that CRPF is an effective therapeutic alternative for unstable proximal humeral fractures.

This was a retrospective clinical study and was approved by the Institutional Review Board of the Fourth Military Medical University and Tangdu Hospital (Xi'an, China). All the participants provided their written informed consent to participate in this study, and the ethics committee approved the consent procedure.

Material and methods

Patients

From May 2009 to April 2013, 38 patients with closed displaced proximal humeral fractures were treated using the CRPF technique at the Department of Orthopedic Surgery, Tangdu Hospital (Xi'an, China). Open reduction and internal fixation (ORIF) treatment was performed when the initial closed reduction failed or the fracture had re-displaced on repeat radiographs taken 5–7 days later. All fractures were stabilized with the percutaneous annulated screw fixation technique by experienced orthopedic surgeons. Out of the 38 patients, 26 were evaluated for their clinical outcomes, and the other 12 patients were excluded: 2 patients changed their address or phone number and could not be contacted, 3 declined participation, 5 suffered closed reduction failure or the fracture had re-displaced and underwent ORIF treatment, and the remaining 2 patients suffered humeral head splitting fractures involving the articular surface, which were not suitable for CRPF treatment. Open fracture injuries were not fit for this therapeutic technique, and were not brought into this study. The 26 participants' general information and fracture types were recorded preoperatively (Table 1).

Table 1. General patient information and fracture types

Details	Number (%)
Age (years)	63.6 (range: 36–79)
< 60 years old	9 (34.6)
> 60 years old	17 (65.4)
Side	left: 11 (42.3) right 15 (57.7)
Gender	female 8 (30.8) male 18 (69.2)
Isolated fracture	20 (76.9)
Multiple fractures	6 (23.1)
Fracture pattern (Neer classification)	2-part 7 (26.9) 3-part 17 (65.4) 4-part 2 (7.7)

Operative planning and surgical technique

The authors' CRPF technique has been described in detail elsewhere but will be reviewed in brief here.[3] All the patients were treated surgically within 2 weeks of the injury (Fig. 1a). The operations were carried out with the patient in a modified beach-chair or supine position with the involved scapula positioned over the edge of the table. Under general anesthesia or nerve-blocking anesthesia, with fluoroscopic control, closed reduction was realized to obtain good fragment contact and alignment. Guide pins were inserted through the deltoid into the humerus towards the humeral head, or crossing from the greater tubercle to the distal fracture end.

Good fragment alignment and the position of the guide pins were confirmed by fluoroscopic imaging. Then 4.5-mm AO/ASIF annulated compression screws were used for fixation along the guide pins. Three to 4 screws were inserted in each case (Fig. 1b). After the insertion of the screws for compression fixation, all of the guide pins were extracted, and each incision was closed with number-5 Ethicon sutures.

Post-operatively, the shoulder was passively moved with gradually improved range. In severely comminuted cases, the shoulder was fixed in a shoulder brace for 3–4 weeks, and then shoulder rehabilitation was performed gradually. The patients were examined in the outpatient clinic

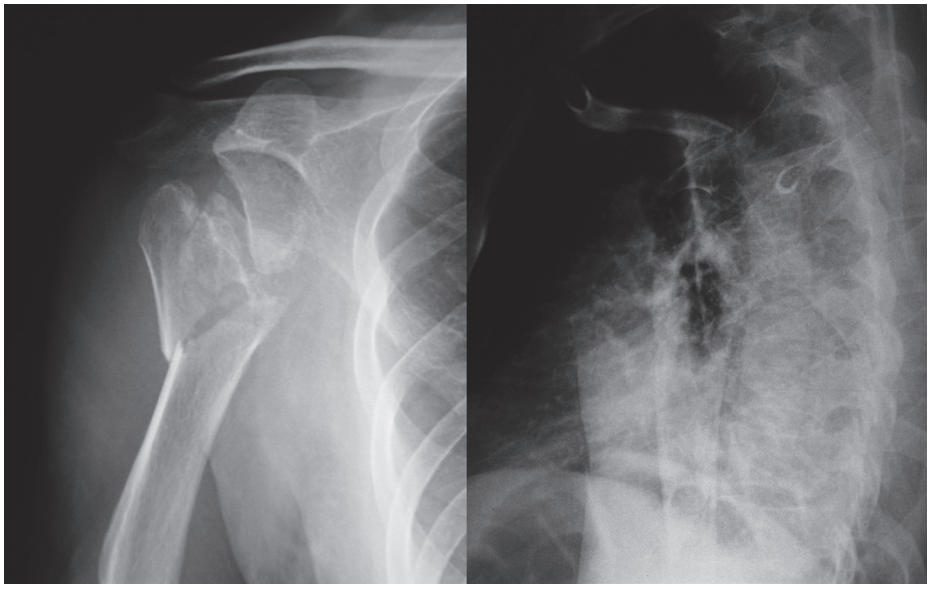

Fig. 1a. Anteroposterior and lateral position radiographs showing a displaced 3-part fracture of the proximal humerus involving the surgical neck and greater tuberosity

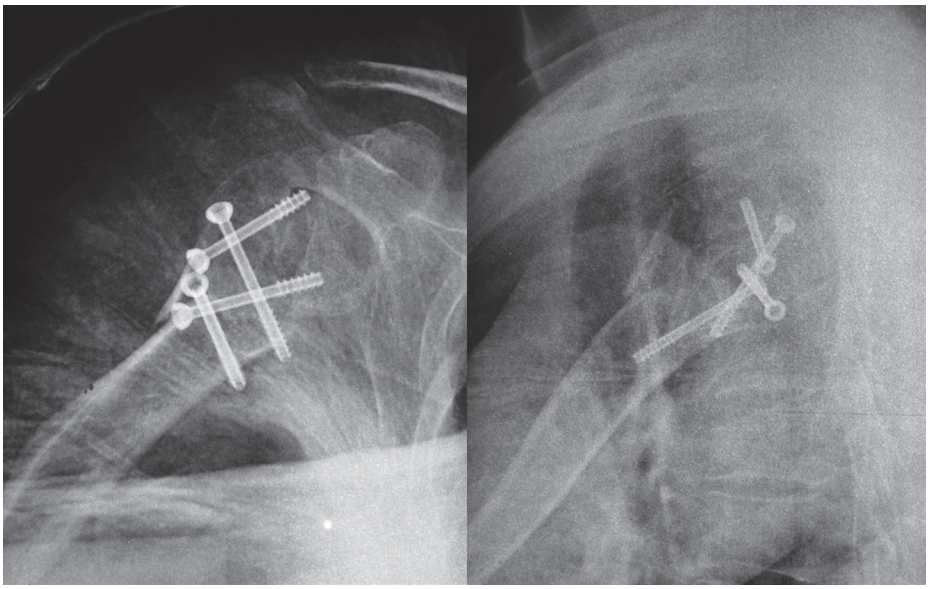

Fig. 1b. Anteroposterior and lateral position radiographs taken immediately after the operation, showing the head and greater tuberosity fragment with alignment restored and fixed with 4 annulated compression screws oriented in different directions

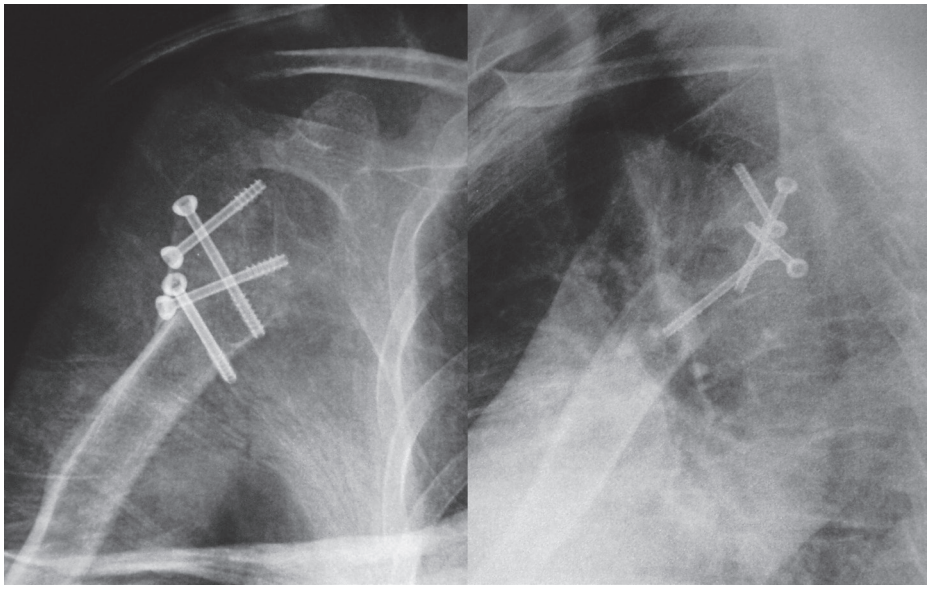

Fig. 1c. AP and lateral position radiographs taken 3 months after the operation, showing the bridging bone between the fragments, which both indicate that bone union has been achieved

3 weeks, 6 weeks, 3 months, and 1–2 years after surgery. The AP position and lateral position were obtained through chest X-rays at each visit to assess alignment, union and any signs of avascular necrosis (Fig. 1c).

Follow-up

A total of 26 patients participated in the follow-up, which ranged from 9 to 24 months (averaging 12.9 months). The patients Constant scores and American Shoulder and Elbow Surgeons (ASES) scores, along with a visual analog scale (VAS) survey, were registered at the 6-month post-operative follow-up and the subsequent follow-up visits when the patients attended an outpatient clinic.

Chest radiographs to check the post-operative AP and lateral position of the shoulder were taken at week 6, week 12 and every subsequent 6th week until bony union was achieved. Bone healing was determined by a combination of painless palpation of the shoulder and radiographic evidence of bridging bone on AP and lateral radiographs.

Radiographic studies

All the patients enrolled in the study had accessible, immediate post-operative radiographs. Radiological bony union was determined by the attending surgeon as at least 3 cortical unions. To score the residual deformity, angulation and displacement were considered independently, and each fragment (the humeral head, greater tuberosity and lesser tuberosity) was scored individually. The final score for each case was the sum of the scores allocated to each fragment. An angulation between 20° and 45° was scored as 1 point and > 45° was scored as 2 points. A displacement between 0.5 and 1 cm was scored as 1 point and > 1 cm was scored as 2 points. If the angulation and displacement were lower than 20° and 0.5 cm, respectively, the quality of the reduction was considered excellent and scored as 0 points.[4] Data are expressed as mean ± standard deviation (SD).

Clinical evaluation

Clinical evaluation of the results was done in accordance with the patients' VAS surveys, Constant scores and ASES scores a minimum of 1 year post-operatively.

Pain was assessed with the VAS, with a maximum of 10 points for this evaluation. Physical examination included measurements of the passive and active ranges of motion of the shoulder with a standard goniometer. Care was taken to prevent compensatory trunk movement during the shoulder range-of-motion measurements.

The Constant score is a 100-point scoring system composed of the following parameters: Pain, daily life activities, range of motion and power. Only the active range of motion was considered. The range of motion was measured and compared with the opposite shoulder.

The ASES questionnaire, which was designed by the research committee of the American Shoulder and Elbow Surgeons in 1994, score totals 100 points and allocates 50 points for measuring function and 50 points for pain. The goal of this questionnaire was to develop a standardized method for evaluating shoulder function based entirely on patient self-evaluation.

Statistical analysis

SPSS software (v. 11.0, SPSS Inc., Chicago, USA) was used for the data analysis. Statistical comparisons of the patients' 6-month post-operative functional scores and the final follow-up were done using Student's t-test, with statistical significance set at $p < 0.05$. Means were compared by use of the Kruskal-Wallis test if the data did not follow a normal distribution. Similarly, comparisons of residual deformity in the post-operative radiographs and the final follow-up were also carried out using Student's t-test.

Results

As shown in Table 2, all the fractures were healed after a follow-up ranging from 9 to 24 months (averaging 12.9 months). There were no superficial or deep wound infections, nor were there any nerve palsies after surgery. There was 1 case of varus abnormality, 2 cases of stiffness of the shoulder joint, 2 cases of ischemic osteonecrosis, 1 case of delayed union, 2 cases suffering from migration of at least one screw (screw removal surgery was performed about 6 months post-operatively, and fortunately no migration of fracture fragments was seen), and 4 cases of traumatic arthritis of shoulder joint.

Table 2. Surgical details of the 26 cases of comminuted proximal humeral fractures

Details	Number (%)
Average time to surgery	6.8 days (ranging 0–16 days)
Pre-operative preparation	traction: 5 (19.2) external fixator or brace: 9 (34.6) emergency operation: 12 (46.2)
Mean operation time	48.5 min (ranging 24–96 min)
Number of implants	2: 3 (11.5) 3: 13 (50.0) 4: 10 (38.5)
Post-operative immobilized time	under 1 week: 5 (19.2) 1–2 weeks: 10 (38.5) 2–3 weeks: 6 (23.1) 3–6 weeks: 3 (11.5) 6 weeks or more: 2 (7.7)
Main complication	varus abnormality: 1 (3.8) joint stiffness: 2 (7.7) ischemic osteonecrosis: 2 (7.7) traumatic arthritis: 4 (15.4) delayed union: 1 (3.8) migration of screws: 2 (7.7)

Clinical evaluations of post-operative follow-up

All fractures healed within a post-operative follow-up period ranging from 9 to 24 months (averaging 12.9 months). The mean interval between the operation and bone union was 14.6 weeks (ranging from 11 to 27 weeks), and the mean time between the operation and fully functional activity was 18.6 weeks (ranging from 15 to 32 weeks). At the final clinical evaluation, shoulder stability was achieved in all the patients, and the mean range of abduction motion was 146.5° (72°–180°) (Table 3).

Table 3. Clinical data of the 26 cases of comminuted proximal humeral fractures

Details	Number
Mean follow-up period	12.9 months (range: 9–24 months)
Mean range of abduction motion	146.5° (range: 72°–180°)
Mean time of bone union	14.6 weeks (range: 11–27 weeks)
Mean time to fully functional activity	18.6 weeks (range: 15–32 weeks)

Radiological evaluations

All cases except for 1 delayed union achieved radiographic union within 3 months. Table 4 shows the degree of residual deformity along with the fracture type according to the Neer classification. No significant differences were observed between the immediate post-operative imaging and the final follow-up imaging.

The statistical analysis demonstrated that the number of fragments displaced in the fracture showed a trend toward correlating with the residual deformity (i.e., the higher the number of fragments, the higher the residual deformity, $p < 0.05$). This means that the higher the severity of the fracture, the worse the quality of reduction and the higher the residual deformity after surgical treatment.

Post-operative functional evaluations

At the 6-month follow-up, the VAS pain score, the Constant score and the ASES score were respectively 1.72 ± 1.24, 73.6 ± 24.8 and 77.4 ± 20.5. At the final follow-up, these 3 scores were respectively 1.62 ± 0.97, 79.5 ± 24.7 and 80.6 ± 15.3. As shown in Table 5, there was no significant difference between the 6-month post-operative score and the final follow-up score in any of the 3 scoring systems.

The VAS was used to measure the average daily pain experienced in the shoulder for each patient. For the purposes of the statistical analysis, the patients were divided into 3 groups based on the Neer classification: 2-part, 3-part and 4-part fractures. The mean VAS pain score for each group was 1.46 for 2-part fractures, 1.78 for 3-part fractures, and 1.95 for 4-part fractures. There was no significant difference in this functional evaluation between the 3 fracture types ($p > 0.05$), or between the 6-month visit and the final follow-up ($p > 0.05$).

The mean Constant score for the entire group was 73.6. At the 6-month follow-up visit the mean Constant score for the 3 fractures types was 75.5 for 2-part fractures, 73.4 for 3-part fractures and 59.2 for 4-part fractures. There was no significant difference in the mean Constant score for the 3 fracture types between the 6-month follow-up visit and the final follow-up ($p > 0.05$), but it decreased

Table 4. Radiological outcomes of the patients

Fracture classification	Post-operative	Final follow-up	p-value
2-part fracture (7 cases)	1.7 ± 1.2	1.5 ± 1.1	> 0.05
3-part fracture (17 cases)	2.3 ± 1.9	2.2 ± 1.8	> 0.05
4-part fracture (2 cases)	3.9 ± 0.7	3.8 ± 0.7	> 0.05
Mean value (26 cases)	2.0 ± 1.5	1.9 ± 1.3	> 0.05

Table 5. Post-operative functional scores of the patients

Fracture classification		Post-operative (6 months)	Final follow-up	p-value
VAS	2-part fracture	1.46 ± 1.13	1.51 ± 1.22	> 0.05
	3-part fracture	1.78 ± 0.94	1.63 ± 1.06	
	4-part fracture	1.95 ± 0.38	1.75 ± 0.28	
Constant score	2-part fracture	75.5 ± 25.8	79.5 ± 23.1	> 0.05
	3-part fracture	73.4 ± 23.5	74.9 ± 18.4	
	4-part fracture	59.2 ± 12.4	60.5 ± 7.21	
ASES score	2-part fracture	79.9 ± 17.3	82.5 ± 12.7	> 0.05
	3-part fracture	76.2 ± 24.2	79.0 ± 17.5	
	4-part fracture	66.8 ± 10.5	68.4 ± 9.3	

from 79.5 to 60.5 ($p < 0.05$) with the increase of fracture fragment numbers.

The ASES score totals 100 points and allocates 50 points for measuring function and 50 points for pain. The format for this questionnaire has several advantages: It is relatively quick to complete, it is simple to score, it can be administered by telephone or by mail/internet, and it is widely accepted by most researchers.[5] In this study, the mean ASES score was also calculated for the aforementioned 3 fracture types; it was 79.9 for 2-part fractures, 76.2 for 3-part fractures and 66.8 for 4-part fractures. The mean ASES score improved from 77.4 to 80.6 ($p > 0.05$) between the 6-month follow-up and the final evaluation.

Discussion

Displaced proximal humeral fractures remain a challenge to orthopedic surgeons, usually resulting in malunion and ischemic osteonecrosis from conservative management or poor shoulder function due to aggressive impairment. In conservative treatment, using an external brace often causes permanent joint stiffness because of the restriction of early functional motion. At the same time, traditional ORIF with insertion of a buttress plate through a curve incision usually requires extensive stripping of the complicated soft tissue, including the pectoralis major insertion, caput longum musculi bicipitis brachii, and even the subscapularis muscle, which can often lead to devascularization of fracture fragments, permanent joint stiffness and increased risks of infection and nonunion.

Recently, the CRPF technique has gained popularity due to improved rigidness, compared with traditional conservative treatment or closed reduction and percutaneous pinning (CRPP) fixation. The compression of fracture fragments from the screw thread provides better fixation intensity, which consequently improves the healing rate, shortens the healing time and decreases ischemic osteonecrosis of the head of the humerus. Based on the results of this paper, for nearly 60% of the patients post-operative immobilized time is no more than 2 weeks, and for more than 80% percent of the patients immobilized time is 3 weeks or under. The main purpose of immobilization is to eliminate post-operative swelling of soft tissue, promote muscle healing and to provide sufficient time for the formation of primary bone callus between the fracture fragments, all of which are requisite conditions for achieving fully functional activity post-operatively. The main treatment goals in proximal humeral fractures are to restore the joint stability, congruity and alignment with minimal soft tissue dissection to allow early joint motion and good shoulder function. As a typical minimally invasive surgical technique, CRPF shows considerable advantages in minimizing soft tissue injury and enabling the patients to perform functional activities much earlier.

Soft tissue preservation is also a very critical issue in the treatment of comminuted proximal humeral fractures, because such a surgical approach with inadequate exposure often involves some important soft tissue injury, such as the axillary nerve, radial nerve, axillary vessels and rotator cuff.[6–8] When percutaneous techniques are used, knowledge of anatomy is very important. Otherwise, even when percutaneous guide pins are inserted, the adjacent neurovascular bundle is susceptible to injury by the guide pins. The guide pins on the lateral cortex should be placed in a safe zone that avoids both the radial and axillary nerves. The radial nerve is relatively safe if the guide pins are kept above the deltoid insertion. The axillary nerve is located an average of 5 cm distal to the acromion; however, it may take a more variable path, particularly the anterior branches.[9] When placing screws through the deltoid, a protective sleeve should be used to decrease the risk of nerve injury. It is also important to ensure that humeral retroversion averages 19°, and percutaneous guide pins or screws must be directed posteromedially to achieve this angle.

In the authors' experience, CRPP is rather a demanding surgical technique. First, it is not very easy to achieve acceptable reduction using indirect reduction techniques. If acceptable alignment cannot be achieved, the technique should be abandoned in favor of a more traditional open reduction.[10] Poor bone quality and fracture comminuting are relative contraindications to this technique. Also, the patients must be cooperative and able to comply with the post-operative protocol and rehabilitation. Therefore, careful patient selection is recommended, as well as close follow-up in the first 4 weeks after surgery to minimize loss of reduction and fixation.

This study also focused on possible operative risks and post-operative complications. The 1st risk is that CRPF is truly technically demanding, and during the operation process, surgeons are exposed to radiation, and may need to try more than once to select very suitable annulated lag screws after the closed reduction and temporary guide pin fixation have been achieved. Of course, the process of placing screws should also be done very carefully, since improper fixing of fragile fragments can lead to further comminuting. Removal of the screws after facture healing is achieved is also quite a difficult process because the percutaneous incisions are usually small and the deltoid muscle is rather strong. The removal operation often requires a longer incision than the therapeutic process; just as everything in the world has 2 sides, a minimally invasive surgical technique will inevitably entail more difficulties during internal fixation removal.

References

1. Carbone S, Tangari M, Gumina S, Postacchini R, Campi A, Postacchini F. Percutaneous pinning of three- or four-part fractures of the proximal humerus in elderly patients in poor general condition: MIROS® versus traditional pinning. *Int Orthop*. 2012;36(6):1267–1273.

2. Chen CY, Chao EK, Tu YK, Ueng SW, Shih CH. Closed management and percutaneous fixation of unstable proximal humerus fractures. *J Trauma*. 1998;45(6):1039–1045.
3. Keener JD, Parsons BO, Flatow EL, Rogers K, Williams GR, Galatz LM. Outcomes after percutaneous reduction and fixation of proximal humeral fractures. *J Shoulder Elbow Surg*. 2007;16(3):330–338.
4. Harrison AK, Gruson KI, Zmistowski B, et al. Intermediate outcomes following percutaneous fixation of proximal humeral fractures. *J Bone Joint Surg Am*. 2012;94(13):1223–1228.
5. Sallay PI, Reed L. The measurement of normative American Shoulder and Elbow Surgeons scores. *J Shoulder Elbow Surg*. 2003;12(6): 622–627.
6. Cheung S, Fitzpatrick M, Lee TQ. Effects of shoulder position on axillary nerve positions during the split lateral deltoid approach. *J Shoulder Elbow Surg*. 2009;18(5):748–755.
7. Rancan M, Dietrich M, Lamdark T, Can U, Platz A. Minimal invasive long PHILOS®-plate osteosynthesis in metadiaphyseal fractures of the proximal humerus. *Injury*. 2010;41(12):1277–1283.
8. Watson M. Major ruptures of the rotator cuff. The results of surgical repair in 89 patients. *J Bone Joint Surg Br*. 1985;67(4):618–624.
9. Sung CM, Roh GS, Sohn HJ, Park HB. Prediction of the location of the anterior branch of the axillary nerve, using correlations with physical factors: a cadaveric study. *J Shoulder Elbow Surg*. 2013;22(11):e9–e16.
10. Röderer G, Erhardt J, Graf M, Kinzl L, Gebhard F. Clinical results for minimally invasive locked plating of proximal humerus fractures. *J Orthop Trauma*. 2010;24(7):400–406.

Neutrophil CD64 as a diagnostic marker for neonatal sepsis: Meta-analysis

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Abstract

Background. Neutrophil CD64 (nCD64) is a promising marker for diagnosing bacterial infections. Several studies have investigated the performance of nCD64 for diagnosing neonatal sepsis and the results are variable. Interest in nCD64 for detecting serious bacterial infections is increasing rapidly.

Objectives. The aim of the present study was to carry out a meta-analysis to systematically evaluate the diagnostic accuracy of nCD64 in neonatal sepsis. As far as the authors know, no previous studies have undertaken this.

Material and methods. A review of studies from Pubmed, Embase and the Cochrane Library, from inception through June 2015, found 7 studies (involving 2213 neonates) fulfilling the inclusion criteria. These 7 studies were subjected to a bivariate meta-analysis of sensitivity and specificity and a summary receiver-operating characteristic (SROC) curve; I^2 was used to test heterogeneity, and the source of heterogeneity was investigated by influence analysis and meta-regression.

Results. The pooled sensitivity and specificity were 80% (95%CI, 69–88%) and 83% (95%CI, 71–90%), respectively. The area under the SROC curve (AUC) was 0.88 (95%CI, 0.85–0.91). The studies had substantial heterogeneity ($I^2 = 87.1\%$).

Conclusions. The results showed that nCD64 is a reliable biomarker for diagnosing neonatal sepsis (AUC = 0.88).

Key words: meta-analysis, neonatal infection, CD64, neonatal sepsis

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Neonatal sepsis is one of the most common causes of morbidity and mortality for neonates all over the world,particularly in developing countries.[1–4] The incidence of neonatal sepsis is approximately 3–40 per 1000 live births, and the mortality rate ranges from 9% to 20%.[5,6]

It is difficult to identify neonatal sepsis early because of a lack of specific clinical manifestations. The signs are hard to distinguish from non-infectious disorders such as maladaptation, respiratory distress syndrome and aspiration syndromes.[7,8] Blood culture is regarded as the reference standard for the identification of serious bacterial infection, but it is time-consuming (2–4 days) and has high false negative/positive rates.[9] This means that broad-spectrum antibiotics are applied to all suspected neonates in case of potential serious outcomes. As a result, drug-resistant strains appear and neonatal healthcare costs escalate.[10]

Several biochemical markers have been studied for the early diagnosis of neonatal sepsis, especially C-reactive protein (CRP) and procalcitonin (PCT). However, the specificity and the value of these markers are not sufficiently reliable. Therefore, a persistent search for better biomarkers of neonatal sepsis is still very necessary.

CD64, a high affinity receptor that binds monomeric IgG, is normally expressed by monocytes and weakly on resting neutrophils.[11] The expression of neutrophil CD64 (nCD64) is considered to be a very early phase of the host's immune response to bacterial infection, increasing about one hour after invasion.[12,13] It is stimulated by inflammatory cytokines, then increases in a graded manner. nCD64 expression remains stable for more than 24 h. The development of flow cytometric technology (FCM) has made it possible to measure nCD64 quickly and precisely with minimal blood volumes.[14,15]

Interest in nCD64 for detecting serious bacterial infections is increasing rapidly. The performance of nCD64 in diagnosing neonatal sepsis has been investigated in several studies and the results are variable.[8,16,17] Taking all the above into consideration, The aim of the present study was to carry out a meta-analysis to systematically evaluate the accuracy of nCD64 in diagnosing neonatal sepsis.

Material and methods

Search strategy and selection criteria

Two investigators systematically searched the PubMed, Embase and the Cochrane Library databases for studies that assessed the accuracy of nCD64 in the diagnosis of neonatal sepsis.

The PubMed and the Cochrane Library combined search term used was (CD64) AND (neonatal sepsis OR neonatal infectious OR sepsis), and the Embase combined search term was (CD64) AND (sepsis). The databases were searched from their inception through June 2015.

A study was considered eligible for inclusion in the present review if it provided data on nCD64 for neonates with or without sepsis. Moreover, nCD64 measurement had to be performed when suspected sepsis presented before antimicrobial therapy. In the septic group, patients had either culture-proven or clinically diagnosed sepsis; in the non-septic group, neonates had benign clinical disorders. Only studies written in English were included.

Furthermore, the studies had to provide sufficient information to construct a 2 × 2 contingency table with false and true positives and negatives provided. All studies that involved healthy neonates and patients older than 28 days were excluded. Animal experiments, reviews, correspondences, case reports, expert opinions and editorials were excluded.

Neonatal sepsis diagnosed in the first 72 h of life was considered early onset sepsis (EOS); after 72 h it was considered late onset sepsis (LOS). The Quality Assessment of Diagnostic Accuracy Studies (QUADAS) tool was used to assess the methodological quality of the studies included.[18] If agreement could not be reached, differences were resolved by a 3rd investigator (ZM).

Data extraction

Two investigators independently extracted the data. If major discrepancies were observed between the data reported in the studies and the data calculated, the corresponding authors were contacted via e-mail with a request for the raw data. If no response was received after sending a reminder, the study was excluded.

The data extracted from the selected studies included the first author; the year of publication; the study design; the number of septic/non-septic patients; the standard of sepsis diagnosis (culture-proven, clinical); the method of nCD64 analysis; the analysis cut-off values; the number of true positive (TP), false positive (FP), false negative (FN) and true negative (TN) specimens; sensitivity/specificity and the positive/negative predictive value (PPV/NPV).

Data analysis

All the studies that evaluated nCD64 in neonates with culture-proven or clinically diagnosed sepsis in comparison with ill neonates that had other conditions were included in the analysis.

A bivariate mixed-effects regression model was performed to synthesize the pooled sensitivity, specificity, positive/negative likelihood ratios (P/N LRs) and the diagnostic odds ratio (DOR). This model did not transform pairs of sensitivity and specificity of individual studies into a single indicator of diagnostic accuracy, but ensured the two-dimensional nature of the data, taking into account any correlations between pairs of studies. A summary receiver-operating characteristic (SROC) curve was also constructed, plotting sensitivity vs specificity, and

the area under the curve (AUC) was calculated. Statistical heterogeneity among the studies was evaluated by I^2 statistics. Values of 25, 50 and 75% for the I^2 test were considered low, moderate and high statistical heterogeneity, respectively. The publication bias of the included studies was assessed by Deek's funnel plot asymmetry test. The Spearman correlation coefficient between the logits of sensitivity and specificity was used to evaluate the presence of a threshold effect in the accuracy of nCD64. Fagan's nomogram was used to calculate post-test probability (PTP). All the above analyses were performed using the Midas Module in Stata software, v. 12 (Stata Corporation, College Station, USA) and Metadisc 1.4 (XI Cochrane Colloquium, Barcelona, Spain). A p-value < 0.05 was considered statistically significant.

Results

Study selection process

The database search retrieved 308 studies. After reviewing the titles and abstracts, 266 articles were excluded, consisting of 98 duplicates, 15 case reports, 93 commentaries, 9 meta-analyses, 13 reviews, 12 meeting abstracts and poster presentations and 26 that did not investigate the diagnostic accuracy of neutrophil CD64 as a marker for sepsis. A further 35 were excluded after a full text review, leaving 7 studies for inclusion.[19–25] The 35 articles included 22 in which the reference group or control group did not correspond to the definitions of the present meta-analysis, 2 that involved adult/pediatric or mixed populations and 13 for which 2 × 2 contingency tables could not be made (Fig. 1).

Characteristics of the studies included

Seven studies were included in the review. The 2213 neonates in these studies came from different parts of the world. Among these 2213 patients, 869 (39%) had sepsis (culture-proven or clinical) and 1344 were non-septic but with other critical conditions. The study population sizes ranged from 32 to 1156. All the studies were carried out in newborn intensive care units (NICUs) and nCD64 expression was measured using flow cytometry analysis. The types of study design were either prospective case-control or cohort studies. The quality of the 7 studies was generally high, satisfying the majority of the QUADAS criteria (Table 1).

Diagnostic accuracy of nCD64

Significant heterogeneity between studies was demonstrated (I^2 = 87.1%) for DOR. The pooled sensitivity of nCD64 for the diagnosis of neonatal sepsis was 80% (95%CI, 69–88%), and the specificity was 83% (95%CI,

Table 1. Characteristics of the studies included in the meta-analysis

Author (year)	Region	Study design	Patients	Prevalence (%)	CD64 analysis	CD64 cutoff	Sensitivity (%)	Specificity (%)	TP	FP	FN	TN	QUADAS score[a]
Du, et al. (2014)[18]	China	prospective case-control study	158	56	FCM	1010 PE bound/cells	81.82	70	72	21	16	49	12
Streimish, et al. (2014)[19]	USA	prospective cohort study	1156	36	FCM	2.42 index[b]	57	75	234	186	177	559	11
Lam, et al. (2013)[20]	China	prospective cohort study	155	35	FCM	5655 PE bound/cell	89	95	49	5	6	95	11
Mazzucchelli, et al. (2013)[21]	Italy	prospective cohort study	32	50	Leuko64 kit	2.85 index	87.5	100	14	0	v2	16	10
Lam, et al. (2011)[22]	China	prospective case-control study	310	44	FCM	6010 PE bound/cell	79	79	107	37	29	137	12
Dilli, et al. (2010)[23]	Turkey	prospective case-control study	109	32	FCM	4.39 index[b]	88.6	85.1	31	11	4	63	9
Bhandari, et al. (2008)[24]	USA	prospective case-control study	293	44	Leuko64 kit	2.3 index[b]	70	62	90	62	38	103	13

[a] QUADAS has a maximum score of 14 indicating the highest quality; [b] Neutrophil CD64 mean equivalent double fluorescence; FCM – flow cytometric technology; TP – true-positive; FP – false-positive; FN – false-negative; TN – true-negative; QUADAS – Quality Assessment for studies of Diagnostic Accuracy.

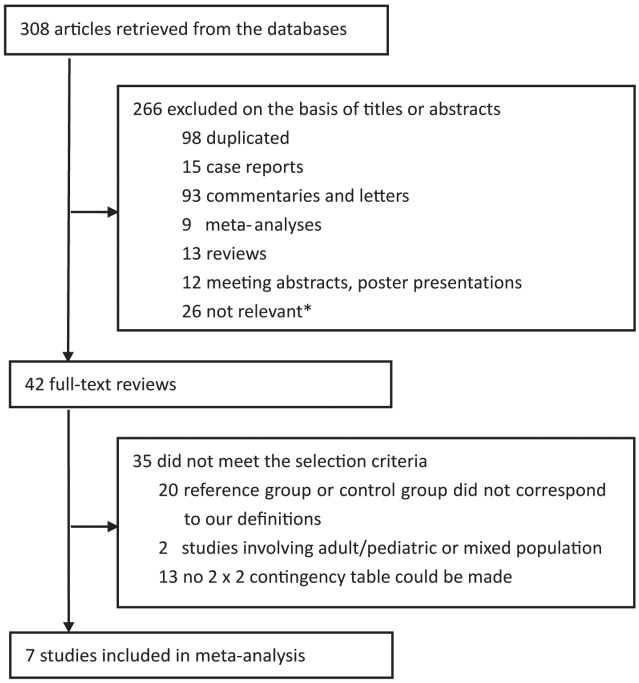

Fig. 1. Study selection. Some studies were excluded for more than one reason
* Did not investigate the diagnostic accuracy of neutrophil CD64 as a marker for sepsis.

71–90%) (Fig. 2). The pooled DOR was 19 (95%CI, 6–57), whereas the pooled P/N LRs were 4.6 (95%CI, 2.5–8.6) and 0.24 (95%CI, 0.14–0.41), respectively. The area under the SROC curve for CD64 was 0.88 (95%CI, 0.85–0.91) (Fig. 3). Fagan's nomogram for likelihood ratios indicated that using nCD64 expression to diagnose neonatal sepsis increased the post-probability to 54% when the results were positive and reduced the post-probability to 6% when the results were negative (Fig. 4). The effect of the diagnostic threshold was not significant (p-value = 0.71 > 0.05). Deek's funnel plot asymmetry test revealed the existence of publication bias with asymmetry in the data (p-value = 0.03 < 0.05) (Fig. 5).

Discussion

Neonatal sepsis is one of the most common causes of neonatal deaths. Diagnosing neonatal sepsis is a serious challenge, because there is no single test that can be used for its early confirmation or exclusion.[14,26] Recently, many researchers have focused on nCD64 as a marker of neonatal sepsis.[8,16,17] In the light of this, the current meta-analysis was undertaken to estimate the efficiency of nCD64 for diagnosing neonatal sepsis.

As noted earlier, PCT is a very promising diagnostic marker of neonatal sepsis.[27] The sensitivity of PCT is 81% and the specificity is 79%. In the present study, the sensitivity and specificity of nCD64 were 80 and 83% respectively, which is similar to PCT. CRP is also an excellent marker and has been applied in clinical practice.[28] The sensitivity of CRP ranges from 30 to 97%, and the specificity ranges from 75 to 100%.[29] In the present meta-analysis, the sensitivity of nCD64 ranges from 57 to 89%, and the specificity ranges from 62 to 100%, indicating that nCD64 is a reli-

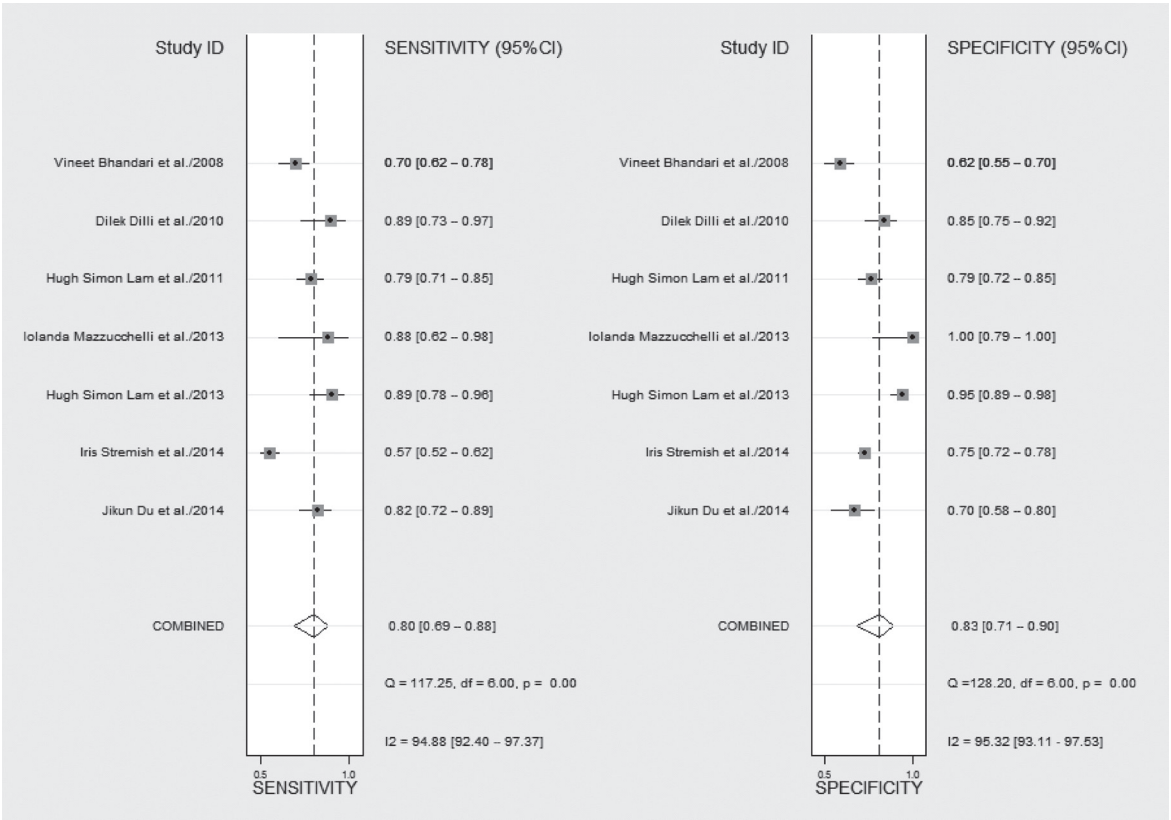

Fig. 2. The sensitivity and specificity of neutrophil CD64 assays for diagnosing neonatal sepsis

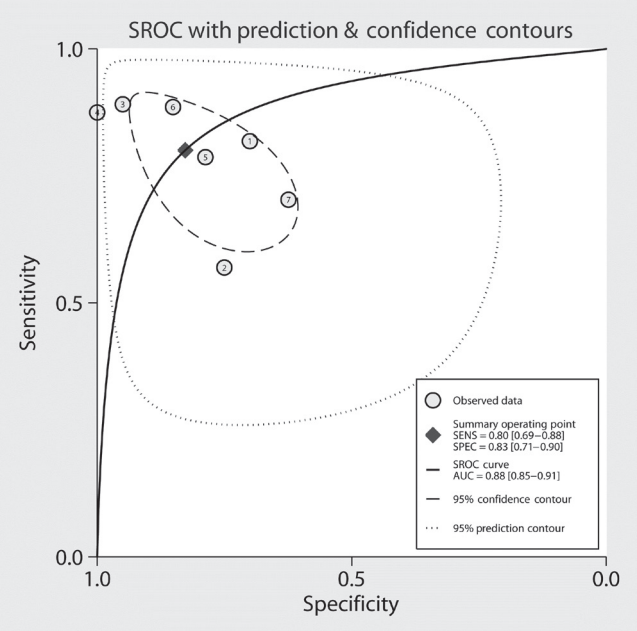

Fig. 3. The summary receiver-operating characteristic curve (SROC) showed a 95% confidence contour and 95% prediction contour

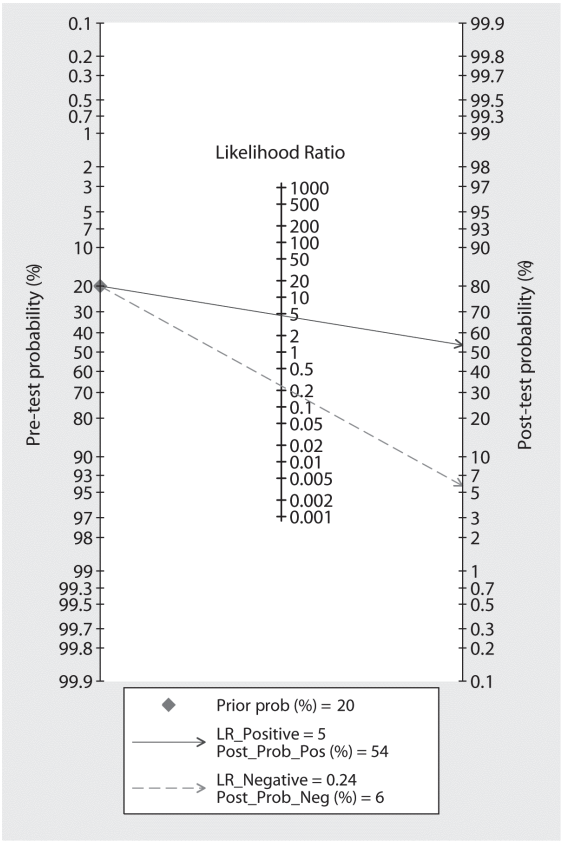

Fig. 4. Fagan's nomogram of the neutrophil CD64 test for diagnosing neonatal sepsis

able marker in the diagnosis of neonatal sepsis. Positive and negative likelihood ratios (P/N LRs) and post-test probability (PTP) are also relevant for clinicians. They both show whether a patient with a positive or negative

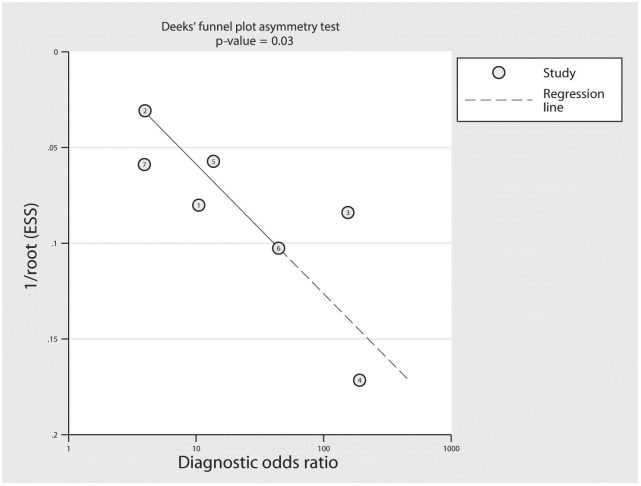

Fig. 5. Deek's funnel plot for the assessment of potential publication bias

test actually has sepsis or not. A PLR of 4.6 indicates that a neonatal with sepsis is 4.6 times more likely to have a positive test result than a neonatal without. The PTP for a positive test result is 54% with a given pretest probability of 20%. Likewise, a NLR of 0.24 reduces the PTP to 6% for a negative result. The area under the SROC curve is 0.88. However, significant statistical heterogeneity exists in the analysis (I^2 = 87.1%). Still, the interpretation of the above findings should not be ignored.

Several methods were tried to find the source of the high heterogeneity, including the threshold effect, publication bias, influence analysis and meta-regression.[30] The different cutoff values for nCD64 did not account for the statistical heterogeneity through the analysis of threshold effect (p = 0.71 > 0.05). Deek's funnel plot asymmetry test showed the existence of publication bias (p-value = 0.03 < 0.05), which is a source of heterogeneity. No valuable information was found through the sensitivity analysis and meta-regression. The meta-regression analysis included study design (prospective cohort or control-case study) and the gestational age of the neonates (preterm or not). The lack of a uniform definition of neonatal sepsis may potentially contribute to the high heterogeneity, especially for the clinically septic but culture-negative newborns, although the concept of clinical sepsis is widely used. This means that the selected studies use different criteria for the definition of sepsis. Thus, the spectrum of disorders and disease varies among the studies included. Considering that age may be a potential source of heterogeneity, studies in which over 15% of the neonates were older than 28 days were excluded. Studies involving healthy neonates as controls in whom nCD64 will not be applied in routine clinical testing were also excluded, so they cannot be representative of the population studied in the current meta-analysis.

This meta-analysis has several limitations. First, substantial heterogeneity was detected among the studies included, but none of the study characteristics accounts

for the majority of this heterogeneity. The studies differ in many ways, especially the definition criteria for neonatal sepsis and the postnatal age of the enrolled neonates. This important limitation will continue to exist in the research in this field until a uniform definition of neonatal sepsis is formulated.[31,32] Second, a wide range of cut-off values in the reported nCD64 tests caused a wide variation in sensitivity and specificity. Even when the same method of measuring nCD64 expression was used, cut-off values were still different.[21,23] Third, publication bias was detected. Studies with satisfactory results are more likely to be published, which can lead to overestimates of diagnostic accuracy. To overcome this problem, the authors searched again for further studies, but could not find additional relevant articles. Finally, only 1 study evaluated the performance of neutrophil CD64 in diagnosing EOS[19] and only 2 evaluated it for LOS.[21,22] The rest of the studies scarcely reported the percentage of EOS and LOS. So the accuracy of nCD64 for the diagnosis of EOS and LOS cannot be assessed.

In conclusion, nCD64 is a helpful marker for diagnosing neonatal sepsis. A study by Dhlamini et al. also shows that nCD64 has a high negative predictive value for excluding neonatal sepsis.[33] But the results of nCD64 tests cannot be used alone to diagnose neonatal sepsis, as neonatal sepsis is a pathophysiological process rather than a specific syndrome and is too complex to be described by a single test. Further studies to determine the optimal cut-off values and to formulate a uniform definition of neonatal sepsis are urgently required.

References

1. Bizzarro MJ, Raskind C, Baltimore RS, et al. Seventy-five years of neonatal sepsis at Yale: 1928–2003. *Pediatrics*. 2005;116(3):595–602. doi: 10.1542/peds.2005-0552.
2. Stoll BJ, Hansen NI, Adams-Chapman I, et al. Neurodevelopmental and growth impairment among extremely low-birth-weight infants with neonatal infection. *Jama*. 2004;292(19):2357–2365. doi: 10.1001/jama.292.19.2357.
3. Lawn JE, Wilczynska-Ketende K, Cousens SN. Estimating the causes of 4 million neonatal deaths in the year 2000. *Int J Epiemilol*. 2006;35(3):706–718. doi: 10.1093/ije/dyl043.
4. Osrin D, Vergnano S, Costello A. Serious bacterial infections in newborn infants in developing countries. *Curr Opin Infect Dis*. 2004;17:217–224.
5. Persson E, Trollfors B, Brandberg LL, et al. Spticaemia and meningitis in neonates and during early infancy in the Göteborg area of Sweden. *Acta Paediatr*. 2002;91:1087–1092.
6. Sundaram V, Kumar P, Dutta S, et al. Blood culture confirmed bacterial sepsis in neonates in a North Indian tertiary care center: Changes over the last decade. *Jpn J Infect Dis*. 2009; 62(1):46–50.
7. Nupponen I, Andersson S, Järvenpää A-L, et al. Neutrophil CD11b expression and circulating interleukin-8 as diagnostic markers for early-onset neonatal sepsis. *Pediatrics*.2001; 108:e12–e12.
8. Layseca-Espinosa E, Pérez-González LF, Torres-Montes A, et al. Expression of CD64 as a potential marker of neonatal sepsis. *Pediatr Allergy Immu*.2002; 13:319–327.
9. Icardi M, Erickson Y, Kilborn S, et al. CD64 index provides simple and predictive testing for detection and monitoring of sepsis and bacterial infection in hospital patients. *J Clinmicrobiol*. 2009;47(12):3914–3919. doi: 10.1128/JCM.00628-09.
10. Ng P. Diagnostic markers of infection in neonates. *Arch Dis Childfetal*. 2004;89:F229–F235.
11. Kolackova M, Kudlova M, Kunes P, et al. Early expression of FcgammaRI (CD64) on monocytes of cardiac surgical patients and higher density of monocyte anti-inflammatory scavenger CD163 receptor in "on-pump" patients. *Mediat Inflamm*. 2007;2008(2008):235461. doi: 10.1155/2008/235461.
12. Hoffmeyer F, Witte K, Schmidt R. The high-affinity FcγRI on PMN: Regulation of expression and signal transduction. *Immunology*. 1997;92:544–552.
13. van der Meer W, Pickkers P, Scott CS, et al. Hematological indices, inflammatory markers and neutrophil CD64 expression: Comparative trends during experimental human endotoxemia. *J Endotoxin Res*. 2007;13(2):94–100. doi: 10.1177/0968051907079101.
14. Ng PC, Lam HS. Diagnostic markers for neonatal sepsis. *Curr Opin Pediatr*. 2006;18:125–131.
15. Nuutila J, Hohenthal U, Laitinen I, et al. Simultaneous quantitative analysis of FcγRI (CD64) expression on neutrophils and monocytes: A new, improved way to detect infections. *H Immunol Methods*.2007;328:189–200.
16. Livaditi O, Kotanidou A, Psarra A, et al. Neutrophil CD64 expression and serum IL-8: Sensitive early markers of severity and outcome in sepsis. *Cytokine*. 2006;36:283–290.
17. Davis BH, Olsen SH, Ahmad E, et al. Neutrophil CD64 is an improved indicator of infection or sepsis in emergency department patients. *Arch Pathol Lab Med*. 2006;130:654–661.
18. Whiting P, Rutjes AW, Reitsma JB,et al. The development of QUADAS: A tool for the quality assessment of studies of diagnostic accuracy included in systematic reviews. *BMC Med Res Methodol*.2003;3:25.
19. Du J, Li L, Dou Y, Li P, et al. Diagnostic utility of neutrophil CD64 as a marker for early-onset sepsis in preterm neonates. *PloS one*. 2014; 9(7):e102647. doi:10.1371/journal.pone.0102647.
20. Streimish I, Bizzarro M, Northrup V, et al. Neutrophil CD64 with hematologic criteria for diagnosis of neonatal sepsis. *Am J Perinat*. 2013;31:021–030.
21. Lam HS, Cheung HM, Poon TCW, et al. Neutrophil CD64 for daily surveillance of systemic infection and necrotizing enterocolitis in preterm infants. *Clin Biochem*. 2013;59:1753–1760.
22. Mazzucchelli I, Garofoli F, Ciardelli L, et al. Diagnostic performance of triggering receptor expressed on myeloid cells-1 and CD64 index as markers of sepsis in preterm newborns. *Pediatr Crit Care Me*. 2013;14:178–182.
23. Lam HS, Wong SPS, Cheung HM, et al. Early diagnosis of intra-abdominal inflammation and sepsis by neutrophil CD64 expression in newborns. *Neonatology*. 2010;99:118–124.
24. Dilli D, Oğuz ŞS, Dilmen Uu, Köker MY, et al. Predictive values of neutrophil CD64 expression compared with interleukin-6 and C-reactive protein in early diagnosis of neonatal sepsis. *J Clin Lab Anal*. 2010;24(6):363–370. doi: 10.1002/jcla.20370.
25. Bhandari V, Wang C, Rinder C, et al. Hematologic profile of sepsis in neonates: Neutrophil CD64 as a diagnostic marker. *Pediatrics*. 2008;121:129–134.
26. Magudumana O, Ballot D, Cooper P, et al. Serial interleukin-6 measurements in the early diagnosis of neonatal sepsis. *J Trop Pediatrics*. 2000;46:267–271.
27. Vouloumanou EK, Plessa E, Karageorgopoulos DE, et al. Serum procalcitonin as a diagnostic marker for neonatal sepsis: A systematic review and meta-analysis. *Intens Care Med*. 2011;37(5):747–762. doi: 10.1007/s00134-011-2174-8.
28. Levy MM, Fink MP, Marshall JC, et al. 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. *Intens Care Med*. 2003;29:530–538.
29. Chan T, Gu F. Early diagnosis of sepsis using serum biomarkers. *Expert Rev Mol Diagn*. 2014;11:487–496.
30. Petitti DB. Approaches to heterogeneity in meta-analysis. *Stat Med*. 2001;20:3625–3633.
31. Committee ACP. Clinical policy for children younger than three years presenting to the emergency department with fever. *Ann Emerg Med*. 2003;42:530–545.
32. Goldstein B, Giroir B, Randolph A. International pediatric sepsis consensus conference: Definitions for sepsis and organ dysfunction in pediatrics. *Pediatr Crit Care Me*. 2005;6:2–8.
33. Dhlamini M, Suchard M, Wiggill T, et al. Neutrophil CD64 has a high negative predictive value for exclusion of neonatal sepsis. *South African of Child Health*. 2013;7:25–29.

Echocardiographic assessment and N-terminal pro-brain natriuretic peptide in hypertensives with metabolic syndrome

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article

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Abstract

Background. N-terminal pro-brain natriuretic peptide (NT-proBNP) release is associated with left ventricular expansion and pressure overload. Elevation of serum levels of natriuretic peptides is observed in patients with impaired as well as preserved left ventricular systolic function. High NT-proBNP has been shown to be related not only to preload but also to increased afterload, especially blood pressure and arterial stiffness.

Objectives. The aim of the study was to evaluate the association of NT-proBNP and echocardiographic parameters in hypertensives with metabolic syndrome.

Material and methods. The study group comprised 133 patients (99 men; mean age 45.9 ± 9.4 years) with at least a 3-month history of arterial hypertension (stages 1 and 2) and fulfilling the diagnostic criteria for metabolic syndrome. Following initial clinical assessment, which included NT-proBNP levels, they underwent two-dimensional echocardiography.

Results. Echocardiographic abnormalities were observed in 60 subjects (45.1%), including left ventricular diastolic dysfunction (LVDdf) in 41 (30.8%) and left ventricular hypertrophy (LVH) in 35 (26.3%). Higher NT-proBNP concentrations were observed in patients with LVH, especially in the presence of LVDdf. Further analysis demonstrated that NT-proBNP correlated negatively with septal E' ($r = -0.38$; $p = 0.015$) and heart rate ($r = -0.42$; $p = 0.006$) in patients with LVDdf, and positively with left ventricular end diastolic diameter ($r = 0.46$; $p = 0.006$) and left ventricular mass index ($r = 0.49$; $p = 0.005$) in subjects with LVH. However, the analysis of ROC curves revealed no NT-proBNP level of good sensitivity and specificity in diagnosing LVDdf/LVH (maximal area under the curve 0.571).

Conclusions. Even a relatively low NT-proBNP concentration can be a useful marker of left ventricular hypertrophy and end-diastolic wall stretch. However, in the present study there was no NT-proBNP level of satisfactory predictive value to diagnose LV abnormalities.

Key words: natriuretic peptides, echocardiography, left ventricular hypertrophy, left ventricular diastolic dysfunction

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N-terminal pro-brain natriuretic peptide (NT-proBNP) is the amino terminal fragment of brain natriuretic peptide that is released from the heart in response to ventricular expansion and pressure overload.[1,2] Elevation of serum levels of natriuretic peptides is observed in patients with impaired as well as preserved left ventricular systolic function, especially those with heart failure.[3,4] However, high NT-proBNP has been shown to be related not only to preload but also to increased afterload, especially high blood pressure and arterial stiffness.[5] In patients with arterial hypertension NT-proBNP has been found to be a marker of impaired diastolic function and left ventricular hypertrophy (LVH).[5,6] Most studies have focused on the relation between natriuretic peptides and echocardiographic measurements, especially left ventricular mass index (LVMI) and markers of left ventricular diastolic dysfunction (LVDdf) derived from mitral flow analysis and tissue Doppler imaging.[6,7,8] Such observations have also been confirmed by magnetic resonance imaging.[9,10] NT-proBNP has also been identified as cardiovascular risk factor.[11,12] However, most of these studies included non-homogenous groups of patients in which co-morbidities could have influenced NT-proBNP assessment.

The aim of the analysis presented in this paper was therefore to evaluate the association of NT-proBNP levels and echocardiographic parameters in patients with essential arterial hypertension and metabolic syndrome.

Material and methods

The study included 133 patients (99 men; mean age 45.9 ± 9.4 years) with at least a 3-month history of arterial hypertension (stages 1 and 2), defined according to the current guidelines.[13] Exclusion criteria were: (1) confirmed secondary arterial hypertension, (2) improperly controlled arterial hypertension with 3 or more medicines, (3) heart failure, (4) cardiomyopathy, (5) significant heart rhythm disorders, (6) significant valvular heart disease, (7) chronic kidney disease, (8) chronic obstructive pulmonary disease, (9) diabetes, (10) polyneuropathy, (11) peripheral vascular diseases, (12) age < 18 years or > 65 years.

All the subjects fulfilled the International Diabetes Federation (IDF) diagnostic criteria for metabolic syndrome,and were recruited from 2 clinical studies performed in the Department of Cardiology and Internal Diseases of the Military Institute of Medicine (Warszawa, Poland) from March 2008 to May 2012.[14] Both studies were conducted according to the Good Clinical Practice guidelines and the Declaration of Helsinki, and had the approval of the local ethics committee (nos. 3/WIM/2008 and 44/WIM/2010). Each patient provided written informed consent to participate in the study. The demographic and clinical characteristics of the study group are shown in Table 1.

Table 1. Basic clinical characteristics

Parameters	Study group (n = 133)
Clinical parameters	
Age [years], mean ± SD	45.9 ± 9.4
BMI [kg/m^2], mean ± SD	32.2 ± 4.1
Male, n [%]	99 (74.4)
SBP [mm Hg], mean ± SD	143.4 ± 16.0
DBP [mm Hg], mean ± SD	91.2 ± 10.1
HR [bpm], mean ± SD	71.7 ± 10.1
Family history of AH, n [%]	79 (59.8)
Controlled AH, n [%]	21 (15.8)
Treated AH, n [%]	68 (51.1)
ACEI, n [%]	39 (29.3)
BB, n [%]	30 (22.6)
CB, n [%]	23 (17.3)
Diuretics, n [%]	42 (31.6)
ARB, n [%]	20 (15.0)
Treated dyslipidemia, n [%]	71 (53.4)
Echocardiography	
LVEDD [mm], mean ± SD	50.1 ± 3.8
LA dimension [mm], mean ± SD	38.4 ± 4.5
LVMI [g/m^2], mean ± SD	96.5 ± 22.0
IVRT [ms], mean ± SD	92.0 ± 13.7
E/A , mean ± SD	1.12 ± 0.28
DT [ms], mean ± SD	184.4 ± 37.7
Septal E', mean ± SD	8.70 ± 2.27
E/E' ratio, mean ± SD	8.84 ± 2.32
LV enlargement, n [%]	2 (1.5)
LA enlargement, n [%]	44 (33.3)
LVDdf, n [%]	41 (30.8)
LVH, n [%]	35 (26.3)
Laboratory tests	
Creatinine [mg/dL], mean ± SD	0.848 ± 0.150
eGFR [mL/min/1.73m^2], mean ± SD	99.1 ± 16.5
FG [mg/dL], mean ± SD	102.8 ± 13.1
T-C [mg/dL], mean ± SD	221.2 ± 47.5
LDL-C [mg/dL], mean ± SD	131.9 ± 39.2
HDL-C [mg/dL], mean ± SD	47.3 ± 14.0
TG [mg/dL], mean ± SD	215.9 ± 116.0
NT-proBNP [pg/mL], mean ± SD	34.5 ± 26.6

A – mitral valve inflow phase A velocity; ACEI – angiotensin converting enzyme inhibitor; AH – arterial hypertension; ARB – angiotensin receptor blocker; BB – beta-blocker; BMI – body mass index; CB – calcium blocker; DBP – diastolic blood pressure; DT – deceleration time; E – mitral valve inflow phase E velocity; eGFR – estimated glomerular filtration rate; FG – fasting glucose; HDL-C – high density lipoproteins; HR – heart rate; IVRT – isovolumetric diastolic time; LA – left atrium; LDL-C – low density lipoproteins; LV – left ventricle; LVDdf – left ventricular diastolic dysfunction; LVEDD – left ventricular end diastolic diameter; LVH – left ventricular hypertrophy; NT-proBNP – N-terminal pro-brain natriuretic peptide; SBP – systolic blood pressure; septal E' – septal annulus early diastolic velocity; T-C – total cholesterol; TG – triglycerides.

Clinical examinations were performed with particular consideration for any family history of arterial hypertension, cardiovascular risk factors and symptoms indicating a secondary cause of arterial hypertension. Office blood pressure measurement (Omron M4 Plus, Kyoto, Japan) was carried out by a technique compliant with the European Society of Cardiology guidelines.[13] Laboratory tests included evaluation of renal function (creatinine, glomerular filtration rate) and metabolic disturbances.

Two-dimensional echocardiography was performed using standard parasternal, apical, and subcostal views (2.5 MHz transducer VIVID 6E GE Medical System, Wauwatosa, USA). The dimension of the left atrium (LA), left ventricular end diastolic diameter (LVEDD) and interventricular septum diameter (IVSD) were measured in the parasternal long-axis view. The left ventricular ejection fraction was calculated according to Simpson's formula, employing a two-dimensional image of the LV chamber during the systole and diastole in the 4- and 2-chamber apical views.

LVH was diagnosed according to the formula recommended by the American Society of Echocardiography (ASE) for estimating LV mass from 2D linear LV measurements and indexed to body surface area (the cut-off values were LVMI > 115 g/m^2 for men and > 95 g/m^2 for women).[15]

Mitral valve inflow was recorded in the apical 4-chamber view with the pulsed-wave Doppler gate positioned in the LV at the level of the mitral valve edges. The E/A ratio and phase E deceleration time were measured. The apical 5-chamber view allowed simultaneous registration of the flow pattern through the aortic and mitral valves and isovolumic diastolic time calculation. Tissue Doppler imaging was performed in the apical views to obtain mitral annular velocity. The sample volume was positioned at or within 1 cm of the septal insertion sites of the mitral leaflets and adjusted as necessary (usually 5–10 mm) to cover the longitudinal excursion of the mitral annulus during diastole. Additionally, mitral septal annulus early diastolic velocity (septal E') was measured and the E/E' ratio was calculated. A diagnosis of LVDdf was based on current guidelines.[15,16] The following values were considered abnormal: left atrium > 40 mm for men and > 38 mm for women, E/A < 0.8, deceleration time > 200 ms, isovolumic diastolic time ≥ 100 ms, septal E' < 8 cm/s, E/E' ratio > 8.

Laboratory tests included fasting glucose, total cholesterol (T-C), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), triglycerides, creatinine, estimated glomerular filtration rate (eGFR, calculated using the MDRD equation) and NT-proBNP, estimated using the Elecsys® proBNP Assay (Roche Diagnostics, Hague Rd., Indianapolis, USA). Only patients with NT-proBNP below 125 pg/mL were included in the statistical analysis.

The statistical analysis was performed using STATISTICA 7.0 software (StatSoft Inc., Tulsa, USA). The distribution and normality of data were assessed by visual inspection and using the Shapiro-Wilk test. Continuous variables were presented as means ± standard deviations (SD); categorical variables were presented as absolute and relative frequencies (percentages). To analyze differences between subgroups, the analysis of variance (ANOVA) was used for normally distributed variables and the Kruskal-Wallis test when the data were not normally distributed. Detailed inter-subgroup comparisons were performed using the appropriate post-hoc tests. For categorical variables, the χ^2 test and Fisher exact test were used. The assessment of the relations between NT-proBNP and other parameters was performed using the Spearman correlation coefficient. A p-value of < 0.05 was taken to indicate statistical significance.

Results

The basic characteristics of the study group are presented in Table 1. Echocardiographic abnormalities were observed in 60 subjects (45.1%), including LVDdf in 41 cases (30.8%, all presenting with impaired relaxation, no pseudonormal or restrictive filling pattern observed), and LVH in 35 cases (26.3%). Left atrium enlargement was observed in 44 subjects (33.3%), and abnormal left ventricular end diastolic diameter in only 2 (1.5%). No significant abnormalities in LV wall contractility, left ventricular ejection fraction or heart valves were observed.

A comparative analysis was performed for 4 subgroups selected on the basis of the presence of LVDdf and/or LVH: Subgroup A consisted of patients without either LVDdf or LVH; subgroup B comprised cases with LVDdf but without LVH; Subgroup C was made up of patients without LVDdf but with LVH; while subgroup D had both LVDdf and LVH. Significant differences between the subgroups were observed for NT-proBNP ($p < 0.05$), higher concentrations of which were observed in the patients with LVH, especially in the presence of LVDdf (subgroup D) – see Fig. 1.

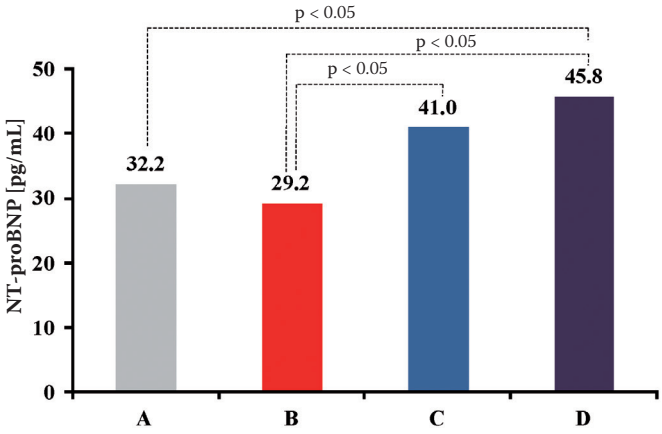
The patients with echocardiographic abnormalities were older and were characterized by higher systolic and diastolic blood pressure. Subgroup B had a higher heart rate, especially in comparison with subgroup C. Left atrium enlargement and higher left ventricular end diastolic diameter were related to LVH, especially in tandem with LVDdf (subgroup D). Only incidental differences were observed for the other parameters assessed (Table 2).

Further analysis demonstrated that NT-proBNP correlated negatively with septal E' ($r = -0.38$; $p = 0.015$) and heart rate ($r = -0.42$; $p = 0.006$) in patients with LVDdf, and positively with left ventricular end diastolic diameter ($r = 0.46$; $p = 0.006$) and LVMI ($r = 0.49$; $p = 0.005$) in subjects with LVH. No significant correlation with other parameters was found (Table 3, Fig. 2).

Table 2. Comparison between subgroups selected on the base of presence of LVDdf and/or LVH

Parameters	Subgroups				p-values	
	A n = 73	B n = 25	C n = 19	D n = 16	ANOVA/ Kruskal-Wallis/ χ^2/Fisher tests	post-hoc tests
Clinical parameters						
Age [years], mean ± SD	43.4 ± 9.8	46.6 ± 8.2	49.2 ± 8.3	52.2 ± 6.1	< 0.05	A vs D*
BMI [kg/m^2], mean ± SD	32.1 ± 3.9	33.3 ± 4.2	30.9 ± 4.6	32.3 ± 3.7	ns	
Male, n [%]	59 (80.8)	19 (76.0)	11 (57.9)	10 (63.5)	< 0.05	A vs C*
SBP [mm Hg], mean ± SD	139.0 ± 14.9	147.5 ± 13.9	145.1 ± 16.2	154.7 ± 17.0	< 0.05	A vs D*
DBP [mm Hg], mean ± SD	89.6 ± 9.3	93.9 ± 11.0	89.5 ± 11.3	96.3 ± 8.9	< 0.05	A vs B* A vs D*
HR [bpm], mean ± SD	71.7 ± 9.3	76.6 ± 10.2	64.8 ± 10.3	72.4 ± 9.1	< 0.05	A vs C* B vs C**
Family history of AH, n [%]	44 (60.3)	16 (64.0)	11 (57.9)	8 (50.0)	ns	
Controlled AH, n [%]	13 (17.8)	3 (12.0)	3 (15.8)	2 (12.5)	ns	
Untreated AH, n [%]	31 (42.5)	11 (44.0)	13 (68.4)	10 (62.5)	< 0.05	A vs C*
Treated AH, n [%]	42 (57.5)	14 (56.0)	6 (31.6)	6 (37.5)	< 0.05	A vs C*
ACEI, n [%]	26 (35.6)	4 (16.0)	3 (15.8)	6 (37.5)	ns	
BB, n [%]	16 (21.9)	7 (28.0)	4 (21.1)	3 (18.8)	ns	
CB, n [%]	13 (17.8)	5 (20.0)	3 (15.8)	2 (12.5)	ns	
Diuretics, n [%]	27 (37.0)	8 (40.0)	2 (10.5)	5 (31.3)	< 0.05	A vs C*
ARB, n [%]	11 (15.1)	7 (28.0)	2 (10.5)	0 (0.0)	< 0.05	B vs D*
Echocardiography						
LVEDD [mm], mean ± SD	49.7 ± 3.6	49.5 ± 3.4	50.9 ± 4.3	51.9 ± 4.2	< 0.05	A vs D* B vs D*
LA dimension [mm], mean ± SD	38.3 ± 2.6	38.5 ± 3.4	39.1 ± 3.5	37.7 ± 9.8	ns	
LV enlargement, n [%]	0 (0.0)	0 (0.0)	1 (5.3)	1 (6.25)	< 0.05	A vs C* A vs D*
LA enlargement, n [%]	19 (26.0)	8 (32.0)	9 (47.4)	8 (50.0)	< 0.05	A vs C* A vs D*
Laboratory tests						
Creatinine [mg/dL], mean ± SD	0.867 ± 0.159	0.824 ± 0.133	0.839 ± 0.138	0.806 ± 0.139	ns	
eGFR [mL/min/1.73 m^2] mean ± SD	99.9 ± 17.8	100.6 ± 15.4	94.7 ± 12.2	98.2 ± 16.4	ns	

* p < 0.05; ** p < 0.01; A – mitral valve inflow phase A velocity; ACEI – angiotensin converting enzyme inhibitor; AH – arterial hypertension; ARB – angiotensin receptor blocker; BB – beta-blocker; BMI – body mass index; CB – calcium blocker; DBP – diastolic blood pressure; DT – deceleration time; E – mitral valve inflow phase E velocity, eGFR – estimated glomerular filtration rate; HR – heart rate; IVRT – isovolumetric diastolic time; LA – left atrium; LV – left ventricle; LVDdf – left ventricular diastolic dysfunction; LVEDD – left ventricular end diastolic diameter; LVH – left ventricular hypertrophy; SBP – systolic blood pressure; septal E' – septal annulus early diastolic velocity.



To evaluate the discriminative power of NT-proBNP as a classifier of echocardiographic abnormalities (LVH and/or LVDdf), the receiver operating characteristic (ROC) was calculated. An analysis of the curves (Fig. 4) revealed that no NT-proBNP level combined both good sensitivity and good specificity (maximal area under the curve: 0.571). The NT-proBNP level with 80% sensitivity was 15.5 pg/mL (with 34.2% specificity), and the level with 80% specificity was 58.8 pg/mL (with 21.7% sensitivity).

Fig. 1. Comparison of NT-proBNP concentration between subgroups selected on the basis of the presence of LVDdf and/or LVH

Table 3. Correlations between NT-proBNP and other parameters in patients with LVDdf (left column) and LVH (right column)

LVDdf (n = 41)			LVH (n = 35)		
NT-proBNP versus					
Parameters	R	p-value	Parameters	R	p-value
Age	0.27	0.086	age	–0.23	0.175
SBP	–0.01	0.943	SBP	0.17	0.335
DBP	0.13	0.420	DBP	0.09	0.596
HR	–0.42	0.006	HR	0.08	0.642
BMI	0.06	0.732	BMI	0.12	0.493
Creatinine	–0.19	0.241	creatinine	–0.18	0.298
eGFR	0.06	0.708	eGFR	0.33	0.055
IVSDD	0.19	0.229	IVSDD	0.26	0.129
LVEDD	0.14	0.400	LVEDD	0.46	0.006
LA dimension	0.21	0.204	LA dimension	0.24	0.170
LVMI	0.23	0.165	LVMI	0.49	0.005
LVEF	–0.09	0.588	LVEF	–0.19	0.289
IVRT	0.25	0.113	IVRT	–0.08	0.633
E/A	–0.29	0.070	E/A	–0.02	0.905
DT	0.16	0.316	DT	0.01	0.958
Septal E′	–0.38	0.015	septal E′	–0.02	0.911
E/E′	0.22	0.169	E/E′	0.06	0.753

A – mitral valve inflow phase A velocity; BMI – body mass index; DBP – diastolic blood pressure; DT – deceleration time; E – mitral valve inflow phase E velocity, eGFR – estimated glomerular filtration rate; HR – heart rate; IVRT – isovolumetric diastolic time; LA – left atrium; LV – left ventricle; LVEDD – left ventricular end diastolic diameter; NT-proBNP – N-terminal pro-brain natriuretic peptide; SBP – systolic blood pressure; septal E′ – septal annulus early diastolic velocity.

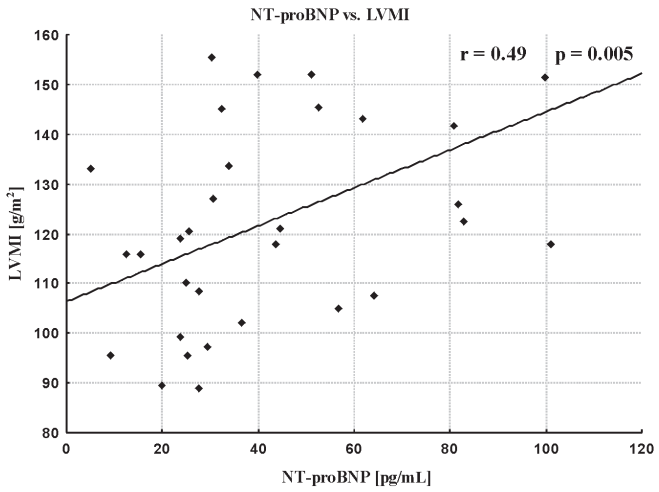


Fig. 2a. Correlations of NT-proBNP with LVMI in patients with LVH

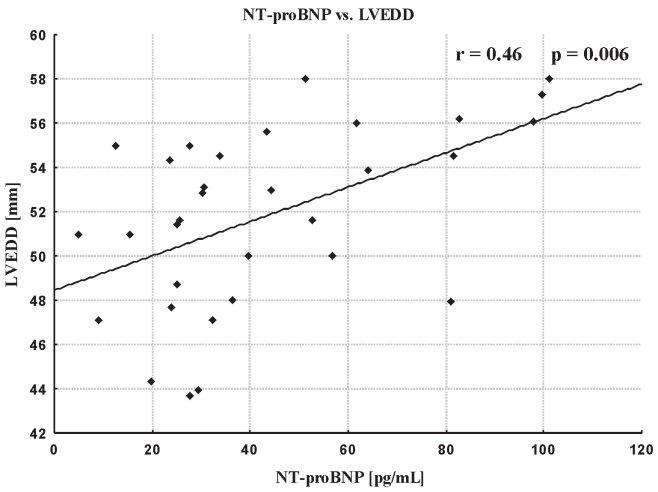


Fig. 2b. Correlations of NT-proBNP with LVEDD in patients with LVH

Discussion

The results of the present study confirmed significant differences between patients with and without LVH and/or LVDdf. The inter-group differences in age, blood pressure and echocardiographic abnormalities are well known and do not require additional comments. Thus, addressing the aim of the study, the discussion focuses on the association of NT-proBNP with hemodynamic and structural echocardiographic parameters.

The analysis revealed that even NT-proBNP values < 125 pg/mL are clinically important and provide pathophysiological information. The results confirmed the association of NT-proBNP release with both LVH and left ventricular filling pattern. The accuracy of low values of natriuretic peptides for detecting abnormalities in heart morphology and hemodynamics has been confirmed in some studies. Mueller et al. defined the NT-proBNP level of 39 pg/mL as highly sensitive (90%) in detecting cardiac structural diseases in echocardiography.[17] Lubien et al.

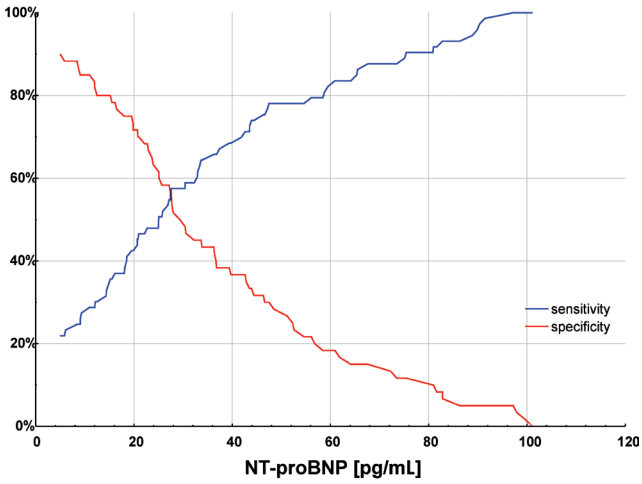


Fig. 3. Sensitivity and specificity curves for NT-proBNP

used a cut-off value of brain natriuretic peptide 62 pg/mL to indentify patients with LVDdf with 85% sensitivity and 83% specificity.[7] Karaca et al. reported even better accuracy – 80% sensitivity and 100% specificity – with a cut-off value of 37 pg/mL.[18] Wei et al. noted the high diagnostic value of brain natriuretic peptide > 40 pg/mL in the assessment of left ventricular end diastolic diameter.[19] The authors of those studies suggested that low natriuretic peptide levels might be able to rule out clinically significant echocardiographic abnormalities.

NT-ProBNP and left ventricular hypertrophy

In the present study patients with arterial hypertension and LVH had higher values of NT-proBNP. Moreover, NT-proBNP correlated positively with left ventricular end diastolic diameter. These observations confirm the association of NT-proBNP concentration with prolonged LV overload and myocyte stretch.[20] These effects are even more apparent in the case of LVH. Goetze et al. confirmed that increased NT-proBNP is independently related to the echocardiographic features of LVH in the general population (3497 patients).[21] A correlation between NT-proBNP and LVMI evaluated by magnetic resonance imaging was also shown by Hildebrandt et al.[9] Moreover, Rivero Otero et al. found that NT-proBNP was elevated in patients with LVH, even in normotensives.[22] Therefore, NT-proBNP, as a biochemical marker of LVMI,[23] appears to be useful in screening for LVH.[4] In contrast, some studies have revealed no association between natriuretic peptides and LVH, especially in the early phase of arterial hypertension.[24] Talwar et al. found that the presence of LVH did not affect NT-proBNP concentrations when LV diastolic function was not impaired.[25] However, the results of the present study show that even relatively low values of NT-proBNP concentration reflect LVMI, supporting its potential role as a marker for LVH.

NT-ProBNP and LVDdf

There was no significant difference in NT-proBNP between subgroups with and without LVDdf when LVMI was not increased (subgroups A and B). This can be explained by the fact that the study group included patients with mild/moderate arterial hypertension and without any severe hemodynamic dysfunction (no subjects with advanced pseudonormal or restrictive filling pattern). However, further analysis revealed that a higher level of NT-proBNP was related to lower septal annulus early diastolic velocity (septal E'), an indicator of LV filling pressure. The association of NT-proBNP and LVDdf in arterial hypertension has been shown in other studies. Mak et al. observed a significant correlation of brain natriuretic peptide level with E/E' (r = 0.48), another marker of LVDdf.[26] Ceyhan et al. found an even more pronounced relationship between NT-proBNP and E/E' (r = 0.80, p < 0.0001), but in a small sample group (n = 40).[2] Those observations might be explained by defective active relaxation and increased stiffness resulting in elevated left ventricular filling pressures. In those patients, higher NT-proBNP release was probably provoked by LV pressure overload and tension. This kind of neurohormonal response can be an early marker of misbalance in ventricular-vascular interactions.[27,28]

None of the patients in the present study reported symptoms specific for heart failure. However, Lubien et al. found that natriuretic peptide levels rose along with diastolic abnormalities, even before the patient had presented with any clinical symptoms.[7] It is known that hypertensive patients with abnormal left ventricular filling patterns are often asymptomatic during daily activities, and become "symptomatic" only when experiencing high levels of exercise stress, when LV pressures increase.[29] Early identification of patients with a higher risk of heart failure seems to be crucial to the prevention of the adverse cardiovascular events.

To sum up, like Bettencourt et al.,[30] the present study found that both LVH and hemodynamic abnormalities are related to higher natriuretic peptide levels. This is not surprising, considering that LVH is a marker of LV remodelling in response to increased wall stress, and LVDdf is its hemodynamic consequence. However, the ROC analysis did not indicate any NT-proBNP level as a classifier with both good sensitivity and high specificity, which limits the use of this simple marker to diagnose LV abnormalities.

Limitations

The authors are aware that the small sample size of the subgroups and the retrospective design are limitations of the study. The low prevalence of LVDdf and LVH could influence the statistical power of the results. Moreover, pharmacotherapy could affect the hemodynamics, heart morphology and laboratory results. Additionally, slight

incidental differences were observed for other parameters (gender distribution, pharmacotherapy). It is worth emphasizing that the concentration on hypertensives with metabolic syndrome limited the potential influence of obesity distribution on the parameters analyzed. These limitations should not create a significant bias, but the results should be considered carefully as relating to a particular group of patients.

Conclusions

Echocardiography and NT-proBNP are shown to be complementary methods in the assessment of hemodynamic disturbances in arterial hypertension. Even relatively low NT-proBNP concentrations can be a useful marker of left ventricular hypertrophy and end-diastolic wall stretch. However, in the present study, there was no NT-proBNP level of satisfactory predictive value to diagnose LV abnormalities.

References

1. McCullough PA, Neyou A. Comprehensive review of the relative clinical utility of B-type natriuretic peptide and N-terminal pro-B-type natriuretic peptide assays in cardiovascular disease. *Open Heart Failure Journal*. 2009;2:6–17.
2. Ceyhan C, Unal S, Yenisey C, Tekten T, Ceyhan FB. The role of N-terminal pro-brain natriuretic peptide in the evaluation of left ventricular diastolic dysfunction: Correlation with echocardiographic indexes in hypertensive patients. *Int J Cardiovasc Imaging*. 2008; 24(3):253–259.
3. McMurray JJ, Adamopoulos S, Anker SD, et al. ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2012: The Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of Cardiology. *Eur Heart J*. 2012;33(14):1787–1847.
4. Vasan RS, Benjamin EJ, Larson MG, et al. Plasma natriuretic peptides for community screening for left ventricular hypertrophy and systolic dysfunction: The Framingham heart study. *JAMA*. 2002; 288(10):1252–1259.
5. Bricca G, Lantelme P. Natriuretic peptides: Ready for prime-time in hypertension? *Arch Cardiovasc Dis*. 2011;104(6–7):403–409.
6. Parekh N, Maisel AS. Utility of B-natriuretic peptide in the evaluation of left ventricular diastolic function and diastolic heart failure. *Curr Opin Cardiol*. 2009;24(2):155–160.
7. Lubien E, DeMaria A, Krishnaswamy P, et al. Utility of B-natriuretic peptide in detecting diastolic dysfunction: Comparison with Doppler velocity recordings. *Circulation*. 2002;105(5):595–601.
8. Santosa YP, Tjandrawati A, Noormartany, et al. Comparison of pro B-natriureticpeptide in hypertensive patients with and without diastolic dysfunction. *Acta Med Indones*. 2008;40(1):19–23.
9. Hildebrandt P, Boesen M, Olsen M, Wachtell K, Groenning B. N-terminal pro brain natriuretic peptide in arterial hypertension: A marker for left ventricular dimensions and prognosis. *Eur J Heart Fail*. 2004;6(3):313–317.
10. Morillas P, Castillo J, Quiles J, et al. Usefulness of NT-proBNP level for diagnosing left ventricular hypertrophy in hypertensive patients: A cardiac magnetic resonance study. *Rev Esp Cardiol*. 2008;61(9): 972–975.
11. Pedersen F, Raymond I, Kistorp C, Sandgaard N, Jacobsen P, Hildebrandt P. N-terminal pro-brain natriuretic peptide in arterial hypertension: A valuable prognostic marker of cardiovascular events. *J Card Fail*. 2005;11:S70–75.
12. Olsen MH, Wachtell K, Tuxen C, et al. N-terminal pro-brain natriuretic peptide predicts cardiovascular events in patients with hypertension and left ventricular hypertrophy: A LIFE study. *J Hypertens*. 2004;22(8):1597–1604.
13. Mancia G, De Backer G, Dominiczak A, et al. 2007 Guidelines for the management of arterial hypertension: The Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). *J Hypertens*. 2007;25:1105–87.
14. Alberti KG, Zimmet P, Shaw J, IDF Epidemiology Task Force Consensus Group. The metabolic syndrome: A new worldwide definition. *Lancet*. 2005;366:1059–1062.
15. Lang RM, Bierig M, Devereux RB, et al. Recommendations for chamber quantification. *Eur J Echocardiogr*. 2006;7(2):79–108.
16. Nagueh SF, Appleton CP, Gillebert TC, et al. Recommendations for the evaluation of left ventricular diastolic function by echocardiography. *Eur J Echocardiogr*. 2009;10(2):165–193.
17. Mueller T, Gegenhuber A, Dieplinger B, Poelz W, Haltmayer M: Capability of B-type natriuretic peptide (BNP) and amino-terminal proBNP as indicators of cardiac structural disease in asymptomatic patients with systemic arterial hypertension. *Clin Chem*. 2005;51(12):2245–2251.
18. Karaca I, Gülcü E, Yavuzkir M, et al. B-type natriuretic peptide level in the diagnosis of asymptomatic diastolic dysfunction. *Anadolu Kardiyol Derg*. 2007;7(3):262–267.
19. Wei T, Zeng C, Chen L, et al. Bedside tests of B-type natriuretic peptide in the diagnosis of left ventricular diastolic dysfunction in hypertensive patients. *Eur J Heart Fail*. 2005;7(1):75–79.
20. Dokainish H. Combining tissue Doppler echocardiography and B-type natriuretic peptide in the evaluation of left ventricular filling pressures: Review of the literature and clinical recommendations. *Can J Cardiol*. 2007;23(12):983–989.
21. Goetze JP, Mogelvang R, Maage L, et al. Plasma pro-B-type natriuretic peptide in the general population: Screening for left ventricular hypertrophy and systolic dysfunction. *Eur Heart J*. 2006;27(24): 3004–3010.
22. Rivera Otero JM, Taléns-Visconti R, Salvador A, et al. Ventricular hypertrophy increases NT-proBNP in subjects with and without hypertension. *Int J Cardiol*. 2004;96(2):265–271.
23. Luchner A, Burnett JC Jr, Jougasaki M, et al. Evaluation of brain natriuretic peptide as marker of left ventricular dysfunction and hypertrophy in the population. *J Hypertens*. 2000;18:1121–1128.
24. Belluardo P, Cataliotti A, Bonaiuto L, et al. Lack of activation of molecular forms of the BNP system in human grade 1 hypertension and relationship to cardiac hypertrophy. *Am J Physiol Heart Circ Physiol*. 2006;291(4):H1529–1535.
25. Talwar S, Siebenhofer A, Williams B, Ng L. Influence of hypertension, left ventricular hypertrophy, and left ventricular systolic dysfunction on plasma N terminal proBNP. *Heart*. 2000;83(3):278–282.
26. Mak GS, DeMaria A, Clopton P, Maisel AS. Utility of B-natriuretic peptide in the evaluation of left ventricular diastolic function: Comparison with tissue Doppler imaging recordings. *Am Heart J*. 2004;148(5):895–902.
27. Cheung BMY, Kumana CR. Natriuretic peptides: Relevance in cardiac disease. *JAMA*. 1998;280:1983–1984.
28. Maeda K, Tsutamoto T, Wada A, Hisanaga T, Kinoshita M. Plasma brain natriuretic peptide as a biochemical marker of high left ventricular end-diastolic pressure in patients with symptomatic left ventricular dysfunction. *Am Heart J*. 1998;135:825–832.
29. Ambrosi P, Oddoze C, Habib G. Utility of B-natriuretic peptide in detecting diastolic dysfunction: Comparison with Doppler velocity recordings. *Circulation*. 2002;106(15):e70.
30. Bettencourt P, Ferreira A, Sousa T, et al. Brain natriuretic peptide as a marker of cardiac involvement in hypertension. *Int J Cardiol*. 1999; 69(2):169–177.

The contribution of clinical assessments to the diagnostic algorithm of pulmonary embolism

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of article

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Abstract

Background. Pulmonary thromboembolism (PE) is a major disease in respiratory emergencies. Thoracic CT angiography (CTA) is an important method of visualizing PE. Because of the high radiation and contrast exposure, the method should be performed selectively in patients in whom PE is suspected.

Objectives. The aim of the study was to identify the role of clinical scoring systems utilizing CTA results to diagnose PE.

Material and methods. The study investigated 196 patients referred to the hospital emergency service in whom PE was suspected and CTA performed. They were evaluated by empirical, Wells, Geneva and Miniati assessments and classified as low, intermediate and high clinical probability. They were also classified according to serum D-dimer levels. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated and evaluated according to CTA findings.

Results. Empirical scoring was found to have the highest sensitivity, while the Wells system had the highest specificity. When low D-dimer levels and "low probabilty" were evaluated together for each scoring system, the sensitivity was found to be 100% for all methods. Wells scoring with a cut-off score of 4 had the highest specificity (56.1%).

Conclusions. Clinical scoring systems may be guides for patients in whom PE is suspected in the emergency department. The empirical and Wells scoring systems are effective methods for patient selection. Adding evaluation of D-dimer serum levels to the clinical scores could identify patients in whom CTA should be performed. Since CTA can only be used conservatively, the use of clinical scoring systems in conjunction with D-dimer levels can be a useful guide for patient selection.

Key words: pulmonary embolism, clinical scoring systems, Wells score

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Pulmonary embolism (PE) is a disease with high morbidity and mortality if not treated, and early diagnosis is of vital importance.[1] However, the symptoms, clinical and laboratory findings have low sensitivity and specificity in confirming a diagnosis of PE.[2] Diagnostic tests to confirm or rule out PE may be expensive and are not always available. Thoracic CT angiography (CTA) is an important method for direct visualization of the embolism, but should be performed selectively in patients in whom PE suspected, because of high radiation and contrast exposure.[3] Other invasive methods such as pulmonary angiography involve the risk of some complications, limiting their routine usage in these patients.[4]

The combination of medical history, physical examination and some simple laboratory parameters have been used to predict the probability of PE.[5,6] Empirical scoring is one of these systems, and it mostly shows the clinical decision of the clinician.[7] The Wells criteria are one of the most objective predictive systems, categorizing patients into low, intermediate and high risk for PE.[8] Geneva scoring is also based on objective variables including arterial blood gas parameters.[9] The Miniati scoring system, another pre-test, has similar results as ventilation-perfusion scintigraphy (V/Q scan) in predicting the presence of PE.[10] After appropriate selection of patients that meet the criteria for further evaluation for PE, these pre-tests can reduce unnecessary imaging and radiation exposure while optimizing PE treatment.[6]

In addition to clinical scoring systems, D-dimer levels are used where deep venous thrombosis (DVT) and PE are suspected. A normal serum D-dimer level may exclude a diagnosis of PE.[11] Clinical scores combined with D-dimer levels have been shown to be useful for excluding PE.[6]

Since clinical scoring systems can serve as a guide for the prediction of PE, the aim of this study was to identify their role in correlation with CTA findings for diagnosing PE.

Material and methods

The study involved 196 patients admitted to the Dokuz Eylul University emergency department in 2009 in whom CTA was performed due to suspected PE. The study was approved by the university ethics committee.

The hospital files of the 196 patients were investigated retrospectively. All the data, including demographics and medical histories, clinical signs and symptoms, laboratory and radiographic test results (arterial blood gas [ABG] analysis, enzyme-linked immunosorbent assay [ELISA], serum D-dimer measurement by the turbidimetric method, electrocardiography [ECG], chest X-ray, lower extremity venous Doppler ultrasound) were recorded. The clinical scores were evaluated according to empirical scoring (Table 1a), the Wells criteria (Table 1b), Geneva scoring (Table 1c) and the Miniati system (Table 1d).[7–10] The patients were classified as low, intermediate or high clinical probability of PE according to their clinical scores, and were also categorized as "PE likely" or "PE unlikely" by a modified Wells score with a cut-off score of 4 points.

Table 1a. Empirical scoring system[7]

High probability	
1	Risk factor present
2	Unexplained dyspnea, tachypne, or pleuritic pain present
3	Unexplained radiographic or gas exchange abnormality present
Intermediate probability	
4	Neither high nor low clinical probability
Low probability	
5	Risk factor not present
6	Dyspnea, tachypnea, or pleuritic pain possibly present but explainable by another condition
7	Radiographic or gas exchange abnormality possibly present but explainable by another condition

Table 1b. Wells scoring system[8]

Clinical signs and symptoms of DVT (min of leg swelling and pain with palpation of the deep veins)	+ 3.0
PE as or more likely than an alternative diagnosis	+ 3.0
Heart rate greater than 100	+ 1.5
Immobilization or major surgery in the previous 4 weeks	+ 1.5
Previous DVT/PE	+ 1.5
Hemoptysis	+ 1.0
Active cancer (treatment ongoing or within the last 6 months or palliative)	+ 1.0

A score of less than 2.0 indicates low pre-test probability, a score between 2.0 and 6.0 indicates intermediate pre-test probability and a score of more than 6.0 indicates high pre-test probability.

Table 1c. Geneva scoring system[9]

Age in years	60–79	+ 1
	≥ 80	+ 2
Previous DVT or PE		+ 2
Heart rate greater than 100/min		+ 1
Recent surgery		+ 3
Pa CO_2, kPa	< 4.8	+ 2
	4.8–5.19	+ 1
Pa O_2, kPa	< 6.5	+ 4
	6.5–7.99	+ 3
	8–9.49	+ 2
	9.5–10.99	+ 1
Chest X-ray	plate-like atelectasis	+ 1
	elevation of hemidiaphragm	+ 1

Geneva Score: 0–4: low probability, 5–9: intermediate probability, > 9: high probability.

were the most frequent symptoms. Tachypnea (42.3%) and tachycardia (14.8%) were the most frequent physical examination findings. Immobilization (41%), the presence of malignancy (18%) and surgical history (16%) were the most frequent risk factors for PE in the study group.

Doppler ultrasound was performed in 51 of 106 patients with suspected deep vein thrombosis (DVT); DVT was confirmed in 21 of the 51 patients (41.2% of those in whom Doppler ultrasound was performed). Transthoracic echocardiography (TTE) revealed abnormal echocardiographic features (seen in PTE) in 9 out of 16 patients in whom TTE was performed.

Arterial blood gas analysis was carried out 88.8% of the patients in the study group; it demonstrated hypoxia and hypocapnia in 55 patients (31.6%), only hypoxia in 48 (27.6%), and only hypocapnia in 43 (24.7%).

Table 1d. Miniati clinical scoring system[10]

High probability
Acute dyspnea, chest pain or syncope (min. one symptom present) not explainable by another condition, with:
Electrocardiographic signs of right heart failure, pulmonary oligemia, amputation of hilar artery or pulmonary infarction (min. 2 findings present)
Intermediate probability
One symptom present without electrocardiographic signs and radiological findings
Low probability
No symptom present or any possible diagnosis (such as COPD or pneumonia) that can be an explanation for these symptoms

The patients' CTA images and the reports of the radiodiagnostics department were reviewed. They were divided into 2 groups according to whether PE was present or not. The patients with positive radiological findings for PE were categorized as the PE group. The parameters of each scoring system were categorized into 2 groups depending on the presence of PE.

The clinical scoring of the patients was analyzed using SPSS 11.0 software (IBM Corp., Armonk, USA). Sensitivity, specificity, positive and negative predictive values were calculated and compared. The combination of a negative D-dimer level and a clinical score of low probability was treated as another group and compared with the rest of the patients regarding the presence or absence of PE. χ^2 statistics were used to compare categorical variables. The statistical significance of any differences was evaluated by the non-parametric Mann-Whitney U-test when the data were from a highly non-normal distribution, and by the T test when the data were from a normal distribution. The area under the reciever operating characteristic (ROC) curve was calculated for each scoring system.

Results

The study group included 107 males (54.6%) and 89 females (45.4%) suspected of PE; their mean age was 64.18 ± 17.44. Dyspnea (73%) and chest pain (45.9%)

D-dimer levels were measured in 141 patients and found to be elevated in 117 patients (83%), with a cut-off level of 500 ng/mL.

PE was diagnosed by CTA in 39 patients (19.9%). The mean age of these patients was 68.85 ± 15.56, and there was no statistically significant difference between ages of the patients in groups with or without PE ($p = 0.062$). The patients with PE included 13 men (33.3%) and 26 women (66.7%). Out of the total 196 patients in whom PE was suspected, 12.1% of the men (13/107) and 29% of the women (26/89) had PE. There was no statistically significant relationship between the presence of PE and gender ($p = 0.064$).

Immobility was the most frequently seen risk factor for PE in patients who were diagnosed with PE by CTA. Multiple predisposing factors for PE were noted in 32.1% of these patients. No risk factor was found to be significant correlated with the presence of PE.

Dyspnea, cough and chest pain were the most frequent symptoms among the PE patients. Fever and syncope were found to be significantly more frequent in the PE group than among patients not diagnosed with PE (Table 2).

Tachycardia, signs of DVT (including pain, swelling and tenderness, etc.) and tachypnea were the most frequent physical examination findings. There was a statistically significant relationship between tachycardia, hypotension, signs of DVT and the presence of PE ($p = 0.0001$, 0.008 and 0.0001 respectively) (Table 2).

Table 2. Symptoms and physical examination findings in relation to the presence of PE

Symptoms and physical examination findings	PE present in CTA (n = 39) (%)	No PE in CTA (n = 157) (%)	p-value
Dyspnea	32/143 (22.4)	111/143 (77.6)	0.108*
Chest pain	13/90 (14.4)	77/90 (85.6)	0.056*
Palpitation	5/29 (17.2)	24/29 (82.8)	0.460*
Hemoptysis	2/12 (16.7)	10/12 (83.3)	0.560*
Fever	8/21 (38.1)	13/21 (61.9)	0.033*
Cough	14/39 (35.8)	25/39 (64.2)	0.530*
Syncope	8/13 (61.5)	5/13 (38.5)	0.001*
Tachypnea	19/83 (22.9)	64/83 (77.1)	0.235*
Tachycardia	28/91 (30.8)	63/91 (69.2)	0.0001*
Hypotension	10/24 (41.7)	14/24 (58.3)	0.008*
DVT sign	20/37 (54.1)	17/37 (45.9)	0.0001*

* χ^2 test.

Table 3. The presence of PE in relation to clinical scores according to the 4 systems

Clinical scoring systems with probability of PE		PE present in CTA (n = 39) (%)	No PE in CTA (n = 157) (%)
Empirical score	high probability	26/29 89.7	3/29 10.3
	intermediate probability	11/80 13.8	69/80 86.3
	low probability	2/87 2.3	85/87 97.7
Wells score	high probability	20/22 90.9	2/22 9.1
	intermediate probability	15/67 22.4	52/67 77.6
	low probability	4/107 3.7	103/107 96.3
Geneva score	high probability	4/12 33.3	8/12 66.7
	intermediate probability	29/94 30.9	65/94 69.1
	low probability	6/90 6.7	84/90 93.3
Miniati score	high probability	3/3 100	0/0 0
	intermediate probability	33/101 32.7	68/101 67.3
	low probability	3/92 3.3	89/92 96.7

Among the patients who had PE, empirical scoring indicated that 26 (89.7%) had a high clinical probability of having PE, 11 (13.8%) had an intermediate probability and 2 (2.3%) had a low clinical probability. According to the Wells scoring criteria, 20 PE patients (90.9%) had a high probability, 15 (22.4%) had an intermediate probability and 4 (3.7%) had a low probability of having PE. According to Geneva scoring only 4 PE patients (3.7%) had a high clinical probability. According to Miniati scoring only 3 patients had a high clinical probability of PE, and all 3 (100%) indeed had PE (Table 3).

The sensitivity of the various scoring systems for PE was found as 94.9% for empirical scoring, 89.7% for the Wells criteria, 84.6% for Geneva scoring, and 92.3% for the Miniati system. The specificity was 54.1, 65.6, 53.5 and 56.7% respectively; PPV was 33.9, 39.3, 31.1 and 34.6 respectively; while NPV was 97.7, 96.3, 93.3 and 96.7% respectively. It was remarkable that empirical scoring had the highest sensitivity, while the Wells method was found to have the highest specificity.

D-dimer levels were analyzed in 141 patients. They were high in 117 patients (83%), with a cut-off value of 500 ng/mL according to the ELISA test. D-dimer sensitivity was found to be 96.3%; specificity was 20.2%; PPV was 22.2% and NPV was 95.3%.

The area under the ROC curve was calculated as 0.76, 0.69, 0.75, 0.74 and 0.82 respectively for the Wells scoring system, Geneva scoring, empirical scoring, Miniati scoring and the Wells system with a cut-off score of 4 (Fig. 1).

Normal serum D-dimer levels in conjunction with low probability scores for PE were evaluated together in every scoring system (Table 4); sensitivity was found to be 100 % in all methods. Wells scoring with a cut-off score of 4 had a specificity of 56.1%. There was no PE in any patient with a normal D-dimer level and a low probability score in any scoring system.

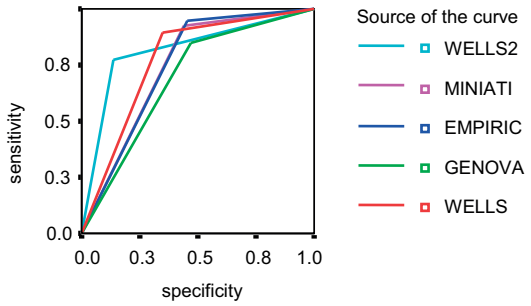

Fig. 1. Scoring systems with the area under the ROC curves. Diagonal segments are produced by ties
Wells2 – Wells system with a cut-off score of 4.

Table 4. The presence of PE according to clinical scores combined with D-dimer levels

Combination of D-dimer levels and scoring systems	PE in BT angiography	
	PE (+)	PE (–)
D-dimer level and empiric score	27	114
The other combinations* Normal D-dimer level and low probability	27 0	101 13
D-dimer level and Wells score	27	114
The other combinations* Normal D-dimer level and low probability	27 0	94 20
D-dimer level and Geneva score	27	114
The other combinations* Normal D-dimer level and low probability	27 0	96 18
D-dimer level and Miniati score	27	114
The other combinations* Normal D-dimer level and low probability	27 0	103 11
D-dimer level and Wells score with a cut off score of 4	19	41
High D-dimer level & Wells score > 4 Normal D-dimer level & Wells score ≤ 4	19 0	18 23

* The other combinations: Normal D-dimer level and intermediate/high probability; high D-dimer level and any probability.

Discussion

Symptoms or physical examination parameters can guide the clinician in the assessment of PE. In the present study, dyspnea and chest pain were the most common symptoms, while tachypnea and tachycardia were the most frequent physical examination findings in patients admitted to the emergency department with suspected PE. These results were similar to other studies.[12] Tachycardia, hypotension and signs of DVT, symptoms like syncope and fever were found to be higher in the PE group, which was statistically significant. Although symptoms and signs are not considered specific for a PE diagnosis, they have an important role in predicting PE.[13]

Immobility, malignancy and recent surgery were found to be the most frequent risk factors for PE.[14] In the present study, there were no statistically significant differences between patients with or without PE regarding predisposing factors. The presence of multiple risk factors (32.1%) in the patients in the study group is thought to be the reason for this result.

As over-investigation of PE in emergency departments still remains an important problem and PE has a high mortality without treatment, early diagnosis is very important. There are some diagnostic procedures, such as pulmonary angiography or CTA which are invasive and expensive, and are not available in all medical centers.[1,15,16] Clinical scoring systems are used to optimize the management of patients with suspected PE and to help select patients who should be examined further for PE. Clinical assessments have been highlighted by several prospective studies. The most important and prominent clinical evaluation systems for PE are the empirical, Wells, Geneva and Miniati pre-tests.

The empirical scoring system is one of the clinical assessments used for evaluating the presence of PE. In one study PE diagnostic rates were found to be 89.9, 11.7 and 2.8% of the cases ranked by empirical scoring as high, intermediate and low probability respectively.[17] In the present study, the PE diagnostic rates were 89.7% of the high probability cases, 13.8% of the intermediate probability cases and 2.3% of the low probability cases. Gülcü et al. found empirical assessment more sensitive and useful than other pre-tests in the diagnostic management of PE.[18] The results of the present study show that that the empirical scoring system had the highest sensitivity and NPV, which makes it an effective method for patient selection for further evaluation with CTA.

The Prospective Investigation of Pulmonary Embolism Diagnosis (PIOPED) study was an important prospective study which found that 68% of the high probability cases, 30% of the intermediate probability cases and 9% of the low probability cases had PE.[12] Wells et al. developed a prediction system by scoring clinical data from the PIOPED study. PE rates were reported to be 66.7%, 20.5% and 3.6% in high, intermediate and low probability groups, respectively, according to the Wells criteria.[2] In the present study, 90.9% of the cases rated high probability according to the Wells system had PE, as did 22.4% of those rated intermediate and 3.7% of those rated low probability. Although some studies have suggested that the Wells score does not guarantee exclusion of PE, especially in older patients, this pre-test has been reported to be more useful than other tests in the diagnosis of PE in respiratory emergencies in most studies.[6,19] In the present study it was remarkable that the Wells method was found to have the highest specificity and PPV of all 4 pre-tests.

The Geneva scoring system had been developed by Wicki et al; 81% of the high probability cases according to Geneva scoring had PE, as did 38% of the intermediate probability cases and 10% of the low probability cases.[9] In one study the Geneva score was reported to be one of the most useful diagnostic tools for PE.[20] The present study showed 33.3, 30.9 and 6.7% rates of PE in the Geneva high, intermediate

and low probability groups respectively, which meant that this scoring system had lower specificity, sensitivity, PPV and NPV than the other 3 scoring systems.

The Prospective Investigative Study of Acute Pulmonary Embolism Diagnosis (PISAPED) study, which used a scoring system called the Miniati method, included acute symptoms of PE, electrocardiographic signs and radiological findings.[10] PE was diagnosed in 91% of the cases classed as high probability according to the Miniati method, 47% of the intermediate probability cases and 9% of the low probability cases.[10] In the present study PE was found in 100% of those the Miniati method rated as high probability, 32.7% of those rated intermediate and 3.3% of those rated low probability. As the 100% high probability comprised only 3 patients, the Miniati scoring system does not seem to be a strong assessment tool.

D-dimer levels are an important test for excluding PE, especially with the help of clinical scoring systems.[12] On their own, D-dimer levels have high sensitivity and NPV, but low specificity and PPV; the low specificity of 35–40% limits the clinical usefulness of D-dimer levels.[20,21] Low D-dimer levels have been used as a guide for excluding patients with low or intermediate probability of suspected PE.[2,12,22] Some studies have shown that the combination of normal D-dimer levels and low clinical probability was safe for the exclusion of PE; van Belle et al. documented a 3-month incidence of venous thromboembolism (VTE) of only 0.5% of after excluding PE with an unlikely clinical score and normal D-dimer levels.[23–26] In the present study the combination of normal serum D-dimer levels and low probability in all the scoring methods had a result of 100% sensitivity, which means no PE was detected in these patients. In the subgroup of patients with normal D-dimer levels and low/intermediate probability, sensitivity was 100% only in the Wells and empirical scoring systems. These results show that normal D-dimer levels and low clinical probability in all systems, and low/intermediate probability in the Wells or empirical scoring methods, are useful combinations for ruling out PE.

Wells et al. updated their scoring system by creating 2 categories (PE likely or unlikely) with a cut-off score of 4 points to improve the simplicity of the method, which was named as modified Wells score.[23] In a previous study, modified Wells scores and D-dimer levels were evaluated together, which was found to be more successful in excluding PE.[23,26,27] In the present study the specificity of the dichotomized Wells system combined with D-dimer levels was 56.1%, which was the highest of all the clinical scoring systems combined with D-dimer levels. This result shows that the dichotomized version of the Wells system combined with D-dimer levels may be an important method for the diagnosis of PE.

The Wells assessment system, which was found to be useful for diagnosis of PE in the present study, is one of the most popular methods currently used in emergency departments. Data showing that the combination of normal serum D-dimer levels and low probability of PE in scoring systems mostly exclude the diagnosis of PE have also been reported in several studies.[23–25] Empirical scoring, which is not as popular as Wells scoring nowadays, was also found to be a useful method for patients with suspected PE. The present study indicates that the empirical method can be used frequently for patient selection in the emergency department.

The present study has some potential limitations. First of all, it is retrospective. The authors believe that it is objective, as patients with the suspicion of PE were included in the study, not only patients with PE. A prospective study in which the patients are followed up for a few months and evaluated with a CTA for the presence of PE may be more objective in comparing the clinical scoring systems. Another limitation was the small number of patients in some of the subgroups.

Conclusions

Clinical scoring systems may be a guide for the diagnostic management of patients in whom PE is suspected. The empirical scoring system and Wells criteria have been found to be the most useful methods for patient selection. Further evaluation of serum D-dimer levels in combination with clinical scores could determine the group of patients in whom CTA should be performed. The combination of normal serum D-dimer levels and low probability scores in any clinical scoring system is a reliable method for the exclusion of PE, which can avoid subjected these patients to CTA. The combination of the dichotomized Wells score and D-dimer level seems to be important in diagnosing PE. Evaluation using clinical scoring systems and D-dimer levels is practical for use in the emergency department to rule out a diagnosis of PE and to guide the diagnostic algorithm for patient selection for further evaluation.

References

1. Dalen JE. Pulmonary embolism: What have we learned since Virchow? *Chest*. 2002;122:1440–1446.
2. Wells PS, Ginsberg JS, Anderson DR, et al. Use of a clinical model for safe management of patients with suspected pulmonary embolism. *Ann Intern Med*. 1998;129:997–1005.
3. Schoepf UJ, Goldhaber SZ, Costello P. Spiral computed tomography for acute pulmonary embolism. *Circulation*. 2004;109:2160–2167.
4. Johnson MS. Current strategies for the diagnosis of pulmonary embolus. *J Vas Interv Radiol*. 2002;13:13–23.
5. Moores LK, Collen JF, Woods KM, Shorr AF. Practical utility of clinical prediction rules for suspected acute pulmonary embolism in a large academic institution. *Thromb Res*. 2004;113:1–6.
6. Tsimogianni AM, Rovina N, Porfyridis I, et al. Clinical prediction of pulmonary embolism in respiratory emergencies. *Thromb Res*. 2011;127(5):411–417.
7. Penaloza A, Mélot C, Dochy E. Comparison of the Wells score with the simplified revised Geneva score for assessing pretest probability of pulmonary embolism. *Thromb Res*. 2007;120(2):173–179. Epub 2006 Oct 20.
8. Hyers TM. Venous thromboembolism. *Am J Respir Crit Care Med*. 1999;159:1–14.

9. Wicki J, Perneger TH, Jumnod A, Bounameaux H, Perrier A. Assessing clinical probability of pulmonary embolism in the emergency ward. *Arch Intern Med*. 2001;161:92–97.
10. Miniati M, Prediletto R, Formichi B, et al. Accuracy of clinical assessment in the diagnosis of pulmonary embolism. *Am J Respir Crit Care Med*. 1999;159:864–871.
11. Perrier A, Desmarais S, Goehring C, et al. D-dimer testing for suspected pulmonary embolism in outpatients. *Am J Respir Crit Care Med*. 1997;156:492–496.
12. The PIOPED Investigators. Value of ventilation/perfusion scan in acute pulmonary embolism: Results of the prospective investigators of pulmonary embolism diagnosis (PIOPED). *JAMA*. 1990;263: 2753–2759.
13. Stein PD, Terrin ML, Hales CA, Palevsky HI, Saltzman HA, Thompson BT. Clinical, laboratory, roentgenographic, and electrocardiographic findings in patients with acute pulmonary embolism and no pre-existing cardiac or pulmonary disease. *Chest*. 1991;100(3):598–603.
14. Anderson FA Jr., Spencer FA. Risk factors for venous thromboembolism. *Circulation*. 2003;107:I9–16.
15. Aydoğdu M, Topbaşi Sinanoğlu N, Doğan NO, et al. Wells score and pulmonary embolism rule out criteria in preventing over investigation of pulmonary embolism in emergency departments. *Tuberk Toraks*. 2014;62(1):12–21.
16. Fedullo PF, Tapson VF. Clinical practice. The evaluation of suspected pulmonary embolism. *N Engl J Med*. 2003;349(13):1247–1256.
17. Ergün P, Oran D, Erdoğan Y, Biber C, Caglar A. Clinical probability and noninvasive methods in the diagnosis of pulmonary thromboembolism. *Solunum Hastalıkları*. 2004;15:8–14.
18. Gülcü A, Akkoçlu A, Yilmaz E, Oztürk B, Osma E, Sengün B. Comparison of clinical assessments with computerized tomography pulmonary angiography results in the diagnosis of pulmonary embolism. *Tuberk Toraks*. 2007;55(2):174–181.
19. Schouten HJ, Geersing GJ, Oudega R, van Delden JJ, Moons KG, Koek HL. Accuracy of the wells clinical prediction rule for pulmonary embolism in older ambulatory adults. *J Am Geriatr Soc*. 2014; 62(11):2136–2141.
20. Blondon M, Le Gal G, Righini M. Diagnostic strategy and comparison of clinical scores for pulmonary embolism. *Rev Med Interne*. 2010;31(11):742–749. Epub 2010 Aug 25.
21. Kelly J, Rudd A, Lewis RR, Hunt BJ. Plasma D-dimers in the diagnosis of venous thromboembolism. *Arch Intern Med*. 2002;162:747–756.
22. Righini M, Aujesky D, Roy PM, et al. Clinical usefulness of D-dimer depending on clinical probability and cutoff value in outpatients with suspected pulmonary embolism. *Arch Intern Med*. 2004; 164(22):2483–2487.
23. Wells PS, Anderson DR, Rodger M, et al. Derivation of a simple clinical model to categorize patients' probability of pulmonary embolism: Increasing the model's utility with the SimpliRED D-dimer. *Thromb Haemost*. 2000;83:416–420.
24. Kruip MJ, Slob MJ, Schijen JH, van der Heul C, Büller HR. Use of a clinical decision rule in combination with D-dimer concentration in diagnostic workup of patients with suspected pulmonary embolism: a prospective management study. *Arch Intern Med*. 2002;162: 1631–1635.
25. Ten Wolde M, Hagen PJ, MacGillavry MR, et al. Non-invasive diagnostic work-up of patients with clinically suspected pulmonary embolism: Results of a management study. *J Thromb Haemost*. 2004;2:1110–1117.
26. Van Belle A, Büller HR, Huisman MV, et al. Effectiveness of managing suspected pulmonary embolism using an algorithm combining clinical probability, D-dimer testing, and computed tomography. *JAMA*.2006;295:172–179.
27. Wolf SJ, McCubbin TR, Feldhaus KM, Faragher JP, Adcock DM. Prospective validation of Wells criteria in the evaluation of patients with suspected pulmonary embolism. *Ann Emerg Med*. 2004;44: 503–510.

L-carnitine treatment of insulin resistance: A systematic review and meta-analysis

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Abstract

Background. L-carnitine has been used for several years as an adjuvant therapy in oxidative stress, blood sugar, high-sensitivity C-reactive protein (CRP), anemia, etc. However, the efficacy of L-carnitine treating insulin resistance (IR) remains controversial. Homeostasis model assessment of Insulin Resistance (HOMA-IR) is widely used in the clinical evaluation of patients with IR.

Objectives. A meta-analysis, including randomized controlled trials (RCTs), was performed to assess the effect of L-carnitine on HOMA-IR patients.

Material and methods. The Cochrane Library, PubMed, and EMBASE databases were systematically searched to identify RCTs which evaluated the effects of L-carnitine on HOMA-IR patients. We screened relevant studies according to predefined inclusion and exclusion criteria. In the selected articles, we extracted the data: study design, sample size, age, L-carnitine dose and regimen, body mass index (BMI) of patients, mode of administration, study duration and study outcomes.

Results. A total of 5 studies were included for the meta-analysis. The result showed L-carnitine was useful in the treatment of IR (WMD –0.724, CI –0.959 –0.488, $p < 0.0001$). Evaluation at 3, 6, 9, 12 months, the p-values were 0.875, 0.165, 0.031, 0, 007, respectively.

Conclusions. L-carnitine was useful in treating patients with IR. L-carnitine can treat IR more effectively with prolonging the medication time. However, more RCTs with long-term L-carnitine treatment of IR are needed to confirm the viewpoint.

Key words: insulin resistance, meta-analysis, L-carnitine

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Many factors can cause insulin resistance (IR), such as infection, acidosis, excessive stress, smoking, exposure to secondhand smoking.[1] IR means the cells require higher concentrations of insulin in order to produce insulin response.[2] Plasma IR, induced by high insulin and high sugar content, can lead to metabolic syndrome, gout and type II diabetes.[3–5]

Homeostasis model assessment of Insulin Resistance (HOMA-IR) was first proposed by Turner of the University of Oxford research group in 1985 and has been widely used in the clinical evaluation of insulin sensitivity.[6] HOMA-IR was calculated as [serum insulin (IU/L) × × Plasma glucose (mmol/L)]/22.5.[6] The HOMA model is used to estimate cell function from fasting plasma insulin and glucose concentrations. A higher score of HOMA-IR indicates more severe IR. HOMA-IR of the normal is less than 2.68.

L-carnitine is an important transporter of long-chain fatty acids which is primary synthesized in the liver and kidney.[7] Its shortage will impair the use of fat as fuel, which will result in an acute metabolic decompensation.[8] Previously, L-carnitine was mainly used to lose weight and to assist in dialyzing kidneys.[9,10] Recently, several studies have reported its use in treating IR.[11,12] However, the real effect of L-carnitine on IR remains unclear. Therefore, we performed this meta-analysis.

Data sources and search strategy

Two investigators (XY and JWJ) independently searched the Cochrane Central Register of Controlled Trials, PubMed, and EMBASE databases from inception to 2014–2010. The search terms were "L-carnitine" and "insulin resistance". If the 2 investigators had disagreements, it would be resolved by a 3rd person (MTL).

Study selection

When selecting studies, we obeyed the following criteria.

Inclusion criteria: Randomized Controlled Trials (RCTs) which evaluated the effects of L-carnitine on HOMA-IR. HOMA-IR as the index in assessing the degree of IR in patients. Treatment time was less than 6 months. Publications written in English.

Exclusion criteria: Non-randomized controlled trials. Results were not evaluated by HOMA-IR. Studies did not offer the detailed data.

Study quality assessment

The Newcastle-Ottawa Scale was used to assess the methodological quality of the included studies based on their selection of participants, comparability of groups and exposure/outcome ascertainment. A total score of 6 or higher (max score, 9) was used to identify higher-quality studies. The quality of these studies was evaluated by 2 authors (XY and JWJ) independently. If 2 investigators had disagreements, it would be resolved by the 3rd person (MTL).

Data extraction and management

The following data was extracted: study design, sample size, mean age of patients, body mass index (BMI) of patients, L-carnitine dose and regimen, mode of administration, study duration and study outcomes. The data was independently extracted by 2 authors (XY and JWJ) and disagreements were resolved by the 3rd author (MTL).

Data synthesis and statistical analysis

Using the data derived from these RCTs, we used the weighted mean differences (WMDs) to report the continuous outcomes, and 95% confidence intervals (CIs) were also calculated.[13] Subgroup analysis was used to assess the differences at evaluation time of 3, 6, 9, 12 months, respectively. I^2 was used to assess the heterogeneity among the selected studies. The meta-analyses were performed with STATA 12.0 software (copyright 1985–2011 StataCorp LP).

Results

Study selection and characteristic

Searching the database with time limit from the database set up date to October 2014. A total of 581 studies were included in the search and then 314 studies based on animal experiments, theme inconsistent, letters, review and meta-analysis were excluded. After reading the full text content roughly, we excluded the papers which were not randomized controlled trial, not relevant to L-carnitine and not relevant to IR. Then, we further analyzed the rest of the data. We further excluded 22 articles which presented with different indicator of IR and units of IR. Finally, 5 studies were included in the meta-analysis.[11,12,14–16] Details of the study selection process and reasons for study exclusion were presented in Fig. 1.

These 5 studies involved a total of 631 patients, in which 319 were treated with L-carnitine and 312 with placebo. Study duration ranged from 4 weeks to 12 months. The characteristics of included studies were summarized in Table 1. In the 5 articles, the Nos score (95% CI) was 6 ± 2. The detail rules of NOS scoring system are listed in Table 2.

Table 1. Characteristics of the included studies

Study	Study design	Patients		Gender (m/f)		BMI		Mean age (years)		HOMA-IR		Route of administration	L-carnitine dose	Study duration	Nos score
		L	C	L	C	L	C	L	C	L	C				
Alessio Molfino (2010)[12]	RCT	8	8	NR	NR	28.6 ± 6.8	25.8 ± 6.8	69.1 ± 12.6	64.2 ± 14.5	1.1 ± 0.5	1.9 ± 0.7	oral	2 g/day	4 weeks	7
Richard Bloomer (2009)[13]	RCT	14	15	NR	NR	27.8 ± 1.7	32.6 ± 24.4	31 ± 3	35 ± 3	2.9 ± 1.2	3.5 ± 1.6	oral	3 g/day	8 weeks	4
Mariano Malaguarnera (2010)[15]	RCT	36	38	20/26	20/18	26.6 ± 3.7	26.5 ± 3.8	47.9 ± 5.4	47.8 ± 5.8	3.26 ± 0.56	4.03 ± 0.71	oral	2 g/day	24 weeks	7
Pamela Maffioli (2010)[16]	RCT	129	125	21/19	21/18	NR	NR	51 ± 4	53 ± 6	7.0 ± 3.7	7.4 ± 4.1	oral	2 g/day	3, 6, 9, 12 months	8
Giuseppe Derosa (2010)[17]	RCT	132	126	20/23	21/25	32.1 ± 2.6	32.2 ± 2.7	NR	NR	6.1 ± 3.2	6.6 ± 3.4	oral	2 g/day	3, 6, 9, 12 months	8

RCT – Randomized Controlled Trial, L – L-carnitine group, C – control group, NR – not reported, BMI – body mass index, HOMA-IR = fasting insulin (IU/l) x fasting plasma glucose (mmoL/L)/22.5, NOS score – The Newcastle-Ottawa Scale (NOS) for assessing the quality.

Table 2. The Newcastle-Ottawa Scale (NOS) tool

NOS assessment	Alessio Molfino (2010)	Richard Bloomer (2009)	Mariano Malaguarnera (2010)	Giuseppe Derosa (2010)	Pamela Maffioli (2010)
Selection (0~4 scores)					
Definition adequate? (1 score)	1	1	1	1	1
Representativeness of the cases? (1 score)	1	1	1	1	1
Selection of controls? (1 score)	1	1	1	1	1
Definition of controls? (1 score)	1	0	1	1	1
Comparability (0~2 scores)					
Select the most important factor? (1 score)	0	0	1	1	1
Any additional factor? (1 score)	1	0	0	0	1
Exposure (0~3 scores)					
Ascertainment of exposure? (1 score)	1	1	1	1	1
Same method of ascertainment for cases and controls? (1 score)	1	0	1	1	1
Non-response rate (1 score)	0	0	0	1	0
Total	7	4	7	8	8

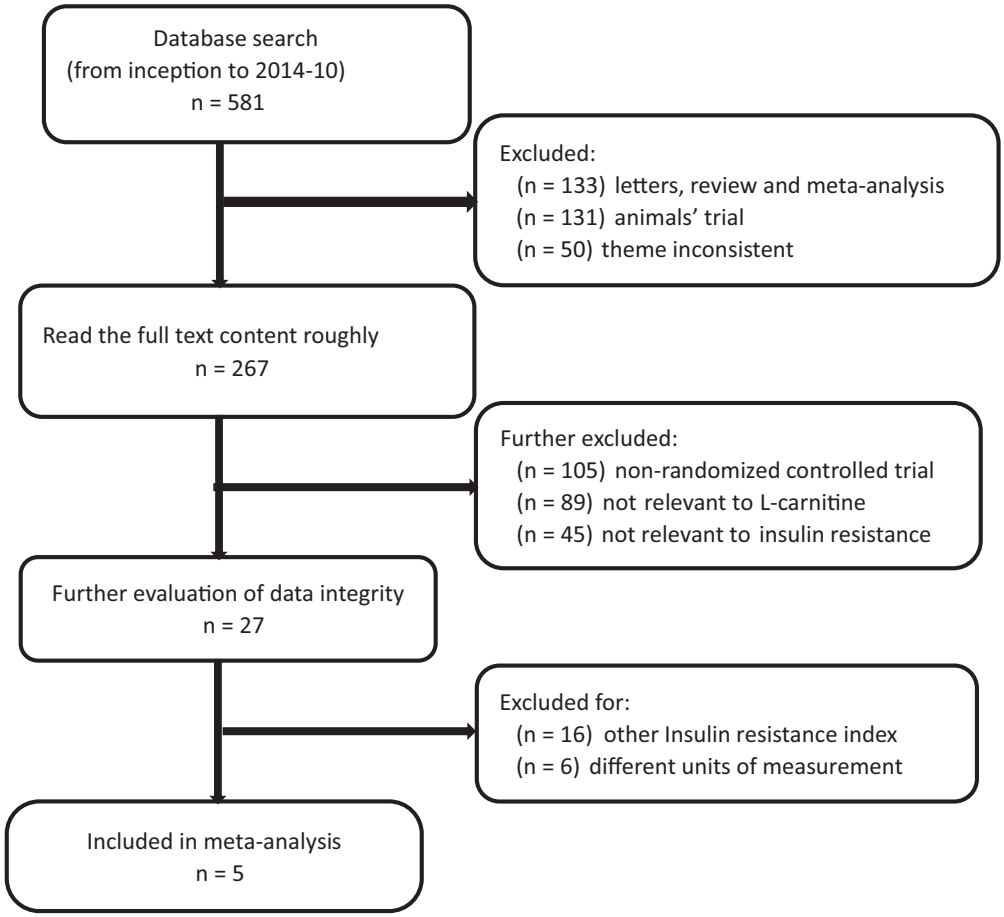

Fig. 1. Flow diagram of literature searching and selecting

Effects of L-carnitine treatment on HOMA-IR

The results showed that L-carnitine could treat HOMA-IR compared to the placebos (WMD –0.724, CI –0.959 TO –0.488, $p < 0.0001$) Fig. 2. Little heterogeneity existed in the analysis ($I^2 = 0\%$). The results of comparisons among different evaluation time indicated that the treatment of L-carnitine became more and more effective with time elapsed (3, 6, 9, 12 months: $p = 0.875$, 0.165, 0.031, 0.007, respectively) Table 3.

Table 3. Different evaluation time comparison chart

Treatment time	WMD [95%CI]	I^2	p-value
3 months	–0.062 (–0.762 to 0.638)	0.0%	0.875
6 months	–0.459 (–1.099 to 0.181)	0.0%	0.165
9 months	–0.201 (–0.385 to –0.018)	0.0%	0.031
12 months	–0.257 (–0.443 to –0.071)	0.0%	0.007

More detailed comparative analysis was conducted, based on 2 of the 5 enrolled literatures, which had longer experimental time. The analysis results showed that the longer the treatment time, the smaller p-value, indicating that with the extension of treatment time, the effect of L-carnitine is more significant.

Discussion

IR is a state in which a given concentration of insulin produces a less-than-expected biological effect.[17] IR widely exists in a variety of diseases not just in diabetes.[8,18] IR makes up a broad clinical spectrum including obesity, glucose intolerance, diabetes, and the metabolic syndrome which will impair the patients' quality of life, lower their survival rate and produce a heavy economic burden on society.[9,15,19,20] However, no therapy has been found to treat IR effectively. Recently, several studies researched the use of L-carnitine in treating IR.[21]

L-carnitine is an important transporter of long-chain fatty acids that can be synthesized endogenously from lysine and methionine or obtained from the diet.[16] L-carnitine is widely used in the maintenance of hemodialysis to

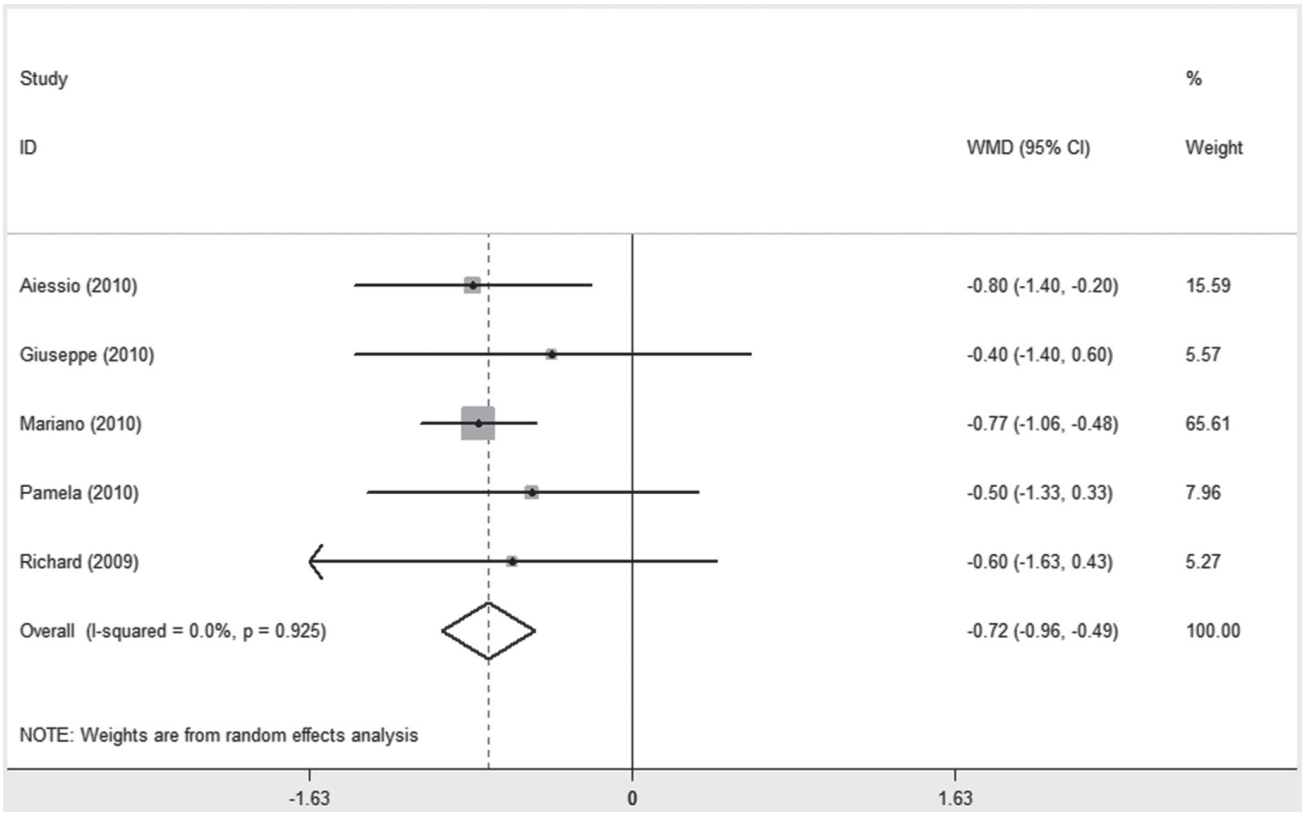

Fig. 2. Forest plot of meta-analysis of L-carnitine on the HOMA-IR in patients

replenish carnitine depletion and has several important proven physiological functions, such as offering nutrition, remedying anemia, adjusting dyslipidemia, curing hyperparathyroidism, developing cognitive functions and enhancing the quality of life in patients.[12,22]

HOMA-IR is a useful index in evaluating IR and is widely used in the evaluation of IR. Several studies showed that HOMA-IR and euglycemic hyperinsulinemic glucose clamps (the IR's gold standard).[23]

Therefore, we performed the meta-analysis to evaluate the effect of L-carnitine on HOMA-IR. Five papers of L-carnitine treating in IR were included in the meta-analysis. The results showed that L-carnitine was useful in reducing the HOMA-IR. Little heterogeneity existed in the analysis. Two of the selected articles included the evaluation time separately at 3, 6, 9, 12 months, then we made a further analysis. The results showed that the treatment of L-carnitine became more and more effective with time elapsed.

The meta-analysis had several advantages. Firstly, the heterogeneity among the included studies was insignificant. Secondly, all of the articles included in the meta-analysis were randomized controlled trials. Thirdly, the articles included in the meta-analysis have high quality by using NOS score tool which has high validity and reliability. Lastly, the paper assessed the efficacy of L-carnitine in 3, 6, 9, 12 months separately.

However, this meta-analysis had some limitations. Firstly, the use of mean ± SD of HOMA-IR was probably unsuitable as it is unlikely to be normally distributed and quartile range seemed to be more suitable. However, all the results in the included studies were expressed as mean ± SD, then we contacted authors to raw data but didn't get a response. Secondly, the experimental time was not long enough in the included studies, and the treatment period did not have uniform criteria. Thirdly, uncontrollable factors may exist in the included 2 groups, such as lifestyle, eating habits and the effects of these factors, which can impact the results significantly. Fourthly, the sample size was small, as only 631 patients were included. This may not represent all the patients well. Lastly, only 2 articles included the evaluation time of 3, 6, 9, and 12 months studies separately and the result may not be persuasive.

Taking all the above into consideration, we need more RCTs with longer treatment time and better control of the interference factors to further research the efficiency of L-carnitine, which could guide clinical practice and provide sound advice for patients more efficiently.

Conclusion

L-carnitine was valid in HOMA-IR. The longer is the treatment duration, the more significant is the treatment effect. However, more RCTs with an adequate sample size and hard endpoints are needed to define the effect of L-carnitine on patients.

References

1. Shulman GI. Cellular mechanisms of insulin resistance. *J Clin Invest*. 2000;106:171–176.
2. Gutch M, Kumar S, Razi SM, Gupta KK, Gupta A. Assessment of insulin sensitivity/resistance. *Indian J Endocrinol Metab*. 2015;19:160–164.
3. Güçlü F, Özmen B, Hekimsoy Z, Kirmaz C. Effects of a statin group drug, pravastatin, on the insulin resistance in patients with metabolic syndrome. *Biomed Pharmacother.* 2004;58:614–618.
4. Shores NJ,Keeffe EB. Is oral l-acyl-carnitine an effective therapy for hepatic encephalopathy? Review of the literature. *Digestive Diseases and Sciences*. 2008;53:2330–2333.
5. Mingrone G. Carnitine in type 2 diabetes. *Ann N Y Acad Sci*. 2004; 1033:99–107.
6. Bonora E, Targher G, Alberiche M, et al. Homeostasis model assessment closely mirrors the glucose clamp technique in the assessment of insulin sensitivity: Studies in subjects with various degrees of glucose tolerance and insulin sensitivity. *Diabetes Care.* 2000;23, 57–63.
7. Mingorance C, Rodriguez-Rodriguez R, Justo ML, Alvarez de Sotomayor M, Herrera MD. Critical update for the clinical use of L-carnitine analogs in cardiometabolic disorders. *Vasc Health Risk Manag*. 2011, 7, 169–176.
8. Takenaka T, Kanno Y, Ohno Y, Suzuki H. Key role of insulin resistance in vascular injury among hemodialysis patients. *Metabolism*. 2007; 56:153–159.
9. Mihalik SJ, Goodpaster BH, Kelley DE, et al. Increased levels of plasma acylcarnitines in obesity and type 2 diabetes and identification of a marker of glucolipotoxicity. *Obesity (Silver Spring).* 2010; 18:1695–1700.
10. Yang SK, Xiao L, Song PA, Xu X, Liu FY, Sun L. Effect of L-carnitine therapy on patients in maintenance hemodialysis: A systematic review and meta-analysis. *J Nephrol*. 2014;27:317–329.
11. Molfino A, Cascino A, Conte C, Ramaccini C, Rossi Fanelli F, Laviano A. Caloric restriction and L-carnitine administration improves insulin sensitivity in patients with impaired glucose metabolism. *J Parenter Enteral Nutr*. 2010;34:295–299.
12. Malaguarnera M, Gargante MP, Russo C, et al. L-carnitine supplementation to diet: a new tool in treatment of nonalcoholic steatohepatitis: A randomized and controlled clinical trial. *Am J Gastroenterol*. 2010;105:1338–1345.
13. Higgins JP and Thompson SG. Quantifying heterogeneity in a meta-analysis. *Stat Med*. 2002;21:1539–1558.
14. Bloomer RJ, Fisher-Wellman KH, Tucker PS. Effect of oral acetyl L-carnitine arginate on resting and postprandial blood biomarkers in pre-diabetics. *Nutr Metab (Lond)*. 2009;6:25.
15. Vaneckova I, Maletinska L, Behuliak M, Nagelova V, Zicha J, Kunes J. Obesity-related hypertension: Possible pathophysiological mechanisms. *J Endocrinol*. 2014;223:R63–78.
16. Derosa G, Maffioli P, At Salvadeo S, et al. Sibutramine and L-carnitine compared to sibutramine alone on insulin resistance in diabetic patients. *Internal Medicine*. 2010;49:1717–1725.
17. Schooneman MG, Vaz FM, Houten SM and Soeters MR. Acylcarnitines: Reflecting or inflicting insulin resistance? *Diabetes*. 2013;62:1–8.
18. Emami Naini A, Moradi M, Mortazavi M, et al. Effects of oral L-carnitine supplementation on lipid profile, anemia, and quality of life in chronic renal disease patients under hemodialysis: A Randomized, double-blinded, placebo-controlled trial. *J Nutr Metab*. 2012;2012:1–6.
19. Biolo G, Stulle M, Bianco F, et al. Insulin action on glucose and protein metabolism during L-carnitine supplementation in maintenance haemodialysis patients. *Nephrol Dial Transplant*. 2008;23:991–997.
20. Malaguarnera M, Vacante M, Bertino G, et al. The supplementation of acetyl-L-carnitine decreases fatigue and increases quality of life in patients with hepatitis C treated with pegylated interferon-alpha 2b plus ribavirin. *J Interferon Cytokine Res*. 2011;31:653–659.
21. Hong ES, Kim EK, Kang SM, et al. Effect of carnitine-orotate complex on glucose metabolism and fatty liver: A double-blind, placebo-controlled study. *J Gastroenterol Hepatol*. 2014;29:1449–1457.
22. Malaguarnera M, Vacante M, Giordano M, et al. Oral acetyl-L-carnitine therapy reduces fatigue in overt hepatic encephalopathy: A randomized, double-blind, placebo-controlled study. *Am J Clin Nutr*. 2011;93:799–808.
23. Donjacour CE, Aziz NA, Overeem S, Kalsbeek A, Pijl H, Lammers GJ. Glucose and fat metabolism in narcolepsy and the effect of sodium oxybate: A hyperinsulinemic-euglycemic clamp study. *Sleep*. 2014; 37:795–801.

The risk of breast cancer due to *PALB2* gene mutations

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D – writing the article; E – critical revision of the article; F – final approval of article

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Abstract

Mutations in the *PALB2* gene are a predisposing factor to the development of breast cancer. *PALB2* gene mutations have been detected in most breast cancer populations, but due to the rarity of their occurrence and the lack of information about their penetrance, they present a challenge when providing genetic counseling for families that have a history of breast cancer. The occurrence rate of *PALB2* mutations ranges from 0.1% to 1.5% depending on the population. Despite the rarity of this mutation, information on the status of *PALB2* mutations in carriers of this gene, as well as for members of their families who may be carriers, is of the utmost importance for clinical reasons, because these mutations are a high risk factor for breast cancer. There is a defined incidence of *PALB2* mutations in patients with breast cancer and negative *BRCA1/BRCA2*. People with a high risk of breast cancer and negative *BRCA1/BRCA2* should be tested for *PALB2* mutations.

Key words: *PALB2*, breast cancer, gene mutation

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PALB2 is a protein that forms a complex with protein *BRCA2*. It functions as an assistant to the *BRCA1* and *BRCA2* proteins in repairing DNA damage, which is part of the body's defense against the development of cancer.[1,2] The *PALB2* protein supports the stabilization of the *BRCA2* protein and specifies its location in a cell's nucleus after DNA damage.[1,3] *BRCA1* regulates *BRCA2* and *PALB2* transfer to the damaged DNA. Cells with diminished *PALB2* protein have disruptions in the *BRCA1*/*BRCA2*-dependent DNA repair path.[4]

Mutations in the *PALB2* gene are a predisposing factor to the development of breast cancer. It has been suggested that *PALB2* mutations, being a high risk factor for breast cancer, are heterozygous germline mutations.[5–7] *PALB2* gene mutations have been detected in multiple breast cancer populations; however, due to the rarity of their occurrence and the lack of information about their penetrance, they present a challenge when providing genetic counseling for families that have a history of breast cancer.[8] Zhang et al. conducted a meta-analysis with the aim of determining the dependencies between the most common mutations of the *PALB2* gene and the risk of breast cancer and concluded that mutations in the *PALB2* gene are associated with an increased risk of breast cancer.[2]

The risk of breast cancer among different populations of women with *PALB2* mutations

The risk of developing breast cancer in women with *PALB2* mutation has been studied by researchers around the world. Dansonka-Mieszkowska et al. conducted research among Polish women.[3] Their study shows that *PALB2* mutations lead to breast cancer in approximately 0.6% of all cases, and they state that the results of other studies are on the same level.[7,9–12] Dansonka-Mieszkowska et al. also discovered a novel deletion in the *PALB2* gene.[3]

PALB2 mutations are mainly related to familial breast cancer.[3,7,11,12] Haanpää et al. note that over the past few years, it has become clear that women with a heterozygous germline mutation in the *PALB2* gene are exposed to an increased risk of breast cancer.[5] According to those authors, among Finnish women the percentage of cases of breast cancer with the occurrence of a *PALB2* mutation is 1%. Bogdanov et al. conducted a study on the occurrence of *PALB*2 mutations in a population of Russian and German women, and reported that a mutation was detected in 4 out 203 patients, which is 2%.[13] Those authors concluded that *PALB2* gene mutations contribute a small percentage to the development of bilateral breast cancer in Russia and Germany. The fact that 2 out of 4 mutations identified in the study were recurrences justifies special screening of cancer patients in Eastern and Central Europe.

The studies mentioned above do not specify the extent of the risk of breast cancer due to a mutation of the *PALB2* gene, which remained unclear until 2014. In 2011, Casadei et al. published a study in which they estimated the risk of breast cancer in carriers of *PALB2* mutations; they reported that the risk is 2 to 3 times higher for women 55 years of age and 3 to 4 times higher for women 85 years of age.[14] In 2013, Nikkila et al. estimated that the risk of developing breast cancer is 6 times greater in cases of *PALB2* heterozygosity.[15] In 2014 Antoniou et al. succeeded in determining the specific risk of breast cancer in individuals with a mutation of the *PALB2* gene.[16] They tested 362 women from 154 families and found that compared to the general population, the risk of breast cancer in people up to 40 years of age with *PALB2* mutation is 8 to 9 times higher; in individuals between 40 and 60 years of age the risk is 6 to 8 times higher; while in people over the age of 60 the risk is 5 times higher. In addition, the authors estimated that the absolute risk of breast cancer in women up to 70 years of age with *PALB2* mutations ranges from 33% for women without family histories of breast cancer to 58% for women with family histories. It is interesting that the risk of breast cancer is higher in women under the age of 40 years and declines in subsequent age groups.

Interestingly, *PALB2* mutations have not been observed in the population of Iceland[17], but they have been detected in most of the populations studied to date. The incidence is low, ranging on average between 1–4%. In some populations, recurrent *PALB2* mutations have been identified; the risk of their occurrence is comparable to the risk of *BRCA* mutation occurrence.[18]

Comparing *PALB2* mutation occurrence in women with familial breast cancer and in sporadic cases

As Smith wrote, "Mutations in the *PALB2* gene are responsible for a small but significant percentage of cancer risks in [familial breast cancer]".[19] Tischkowitz et al. conducted a study to determine the risk of breast cancer in women whose relatives had *PALB2* mutations and in those whose family members did not carry this mutation; the results indicated that the risk was higher in the women in the first group.[20] In the study by Haanpää et al. mentioned earlier, 3.6% of 56 patients at high risk for breast cancer had *PALB2* mutations.[5] Those authors believe that mutations are more common in women with familial breast cancer. Based on this, they assert that *PALB2* gene mutation testing should be incorporated into routine practice in Finland; they also suggest that *PALB2* gene mutations are common enough that routine testing would be valuable worldwide. Janatova et al. found 16 cases of *PALB2*

mutation in a total of 409 women from the Czech Republic, and a high incidence (5.5%) occurred in women with a family history of breast cancer.[21] Hartley et al. conducted a study that confirmed that while *PALB2* mutations are rare, an increase in the number of cases of breast cancer in a family increases the likelihood of *PALB2* mutations as well.[8] Women with 3 or more cases of breast cancer in the family have a 2.6% likelihood of having a *PALB2* mutation. Extensive research was carried out by Southley et al. on Australian women, both with and without breast cancer.[22] They selected women in whose families *PALB2* mutations occurred as well as those without *PALB2* mutations. The objective of the study was to assess the occurrence rate and penetrance of these mutations in Australian women. No *PALB2* mutations were found in the healthy women. Eight women (1%) from the group with breast cancer or *PALB2* mutations in the family also had *PALB2* mutations; among women with breast cancer and no family history of *PALB2* mutations, *PALB2* mutations occurred in 5 (0.4%) cases. The authors concluded that PALB2 protein tests are justified when women are carriers of the *BRCA1* and *BRCA2* genes. In the case of women and family members who are carriers of the *PALB2* gene it is sufficient to provide reasonable prevention, screening and clinical management. Heikkinen et al. conducted a study in southern Finland in which they found *PALB2* mutations in 19 out of 947 women (2%) with familial breast cancers and in 8 out of 1274 (0.6%) women with sporadic breast cancers.[23] The authors also noted that carrying a *PALB2* mutation is connected with a shorter lifespan, especially in patients with familial breast cancer. However, Fernandes et al. conducted a study showing that 69 out of 1479 patients (4.7%) were carriers of *PALB2* mutations; 1.05% of the patients with familial breast cancer had *PALB2* mutations, whereas among the low-risk patients the percentage amounted to 0.38%.[24]

PALB2 mutations with negative *BRCA1* and *BRCA2*

Many researchers have taken an interest in the question of *PALB2* mutations without *BRCA1* and *BRCA2* mutations and the possibilities of introducing tests for *PALB2* into routine practice. Blanco et al. investigated the incidence of mutations in *PALB2* patients with breast cancer and negative *BRCA1*/*BRCA2,* both with and without a history of pancreatic cancer in the family.[25] Those authors found that the frequency of *PALB2* mutations in their study group was 1.5%. According to them, previous studies indicated that the frequency of *PALB2* mutations in breast cancer families ranges from 0%[26,27], through 2.1%[28] up to 4.8% [29] Haanpää et al. stated that people with high risk of breast cancer and negative *BRCA1*/*BRCA2* should be tested for *PALB2* mutations.[5] According to them, these mutations are common enough that a single test for people with a high risk of cancer and negative *BRCA1*/*BRCA2* should be implemented around the world. Hartley et al. have the same opinion, considering *PALB2* mutations a "small but significant" number of all mutations in women in breast cancer families with negative *BRCA1*/*BRCA2*.[8] Janatova et al. also pointed out that *PALB2* gene mutation analysis is recommended for patients with hereditary breast cancer when *BRCA1* and *BRCA2* are negative, because of high frequency of *PALB2* mutations occurring in those patients.[21]

Another study

Teo et al. compared cancer morphology with and without *PALB2* mutations, evaluating 28 cases of women with breast cancer with *PALB2* mutations and 828 cases without *PALB2* mutations, among which 58 women with breast cancer had *BRCA1* and *BRCA2* mutations.[6] Based on the results, the authors concluded that cancers with *PALB2* mutations demonstrate sclerosis and that this distinguishes them from other groups of cancers.

Summary

PALB2 mutations are rare; their occurrence rate ranges from 0.1% to 1.5% depending on the population.[3,6,7,9,10,13,15,21] Despite the rarity of these mutations, information on the status of *PALB2* mutations in carriers of this gene, as well as for the members of their families that may be carriers, is of the utmost importance for clinical reasons, because these mutations are a high risk factor for breast cancer. Identifying carriers of *PALB2* mutations means appropriate tests can be conducted and appropriate treatment can be selected. Among those therapies are some that allow repair dysfunctions of homologous DNA to be pinpointed.[30] Because of the risk of breast cancer recurrence in patients with *PALB2* mutations[20], awareness of the presence of these mutations can lead to earlier detection of cancer and reduce the required treatment. The introduction of integrated *PALB2* testing to clinical practice is still a work in progress, and identifying carriers of *PALB2* mutations can help to facilitate this process.[6] Determination of PALB2 status is also important because of cases of negative *BRCA1* and *BRCA2* in women with diagnosed breast cancer.

References

1. Xia B, Sheng Q, Nakanishi K, et al. Control of BRCA2 cellular and clinical functions by a nuclear partner, PALB2. *Mol Cell.* 2006;22: 719–729.
2. Zhang Y, Wang X, Kang S, Li X, Geng J. Common variants in the PALB2 gene confer susceptibility to breast cancer: A meta-analysis. *Asian Pac J Cancer Prev.* 2013;14:7149–7154.
3. Dansonka-Mieszkowska A, Kluska A, Moes, et al. A novel germline PALB2 deletion in Polish breast and ovarian cancer patients. *Medical Genetics.* 2010;11(20):1–9.

4. Zhang F, Ma J, Wu J, Ye L, et al. PALB2 links BRCA1 and BRCA2 in the DNA-damage response. *Curr Biol.* 2009;19:524–529.
5. Haanpää M, Pylkäs K, Jukka S Moilanen JS, Winqvist R. Evaluation of the need for routine clinical testing of PALB2 c.1592delT mutation in BRCA negative Northern Finnish breast cancer families. *Medical Genetics*. 2013;14(82):1–6.
6. Teo ZL, Provenzano E, Dite GS, et al. Tumour morphology predicts PALB2 germline mutation status. *Br J Cancer.* 2013;109(1):154–163.
7. Rahman N, Seal S, Thompson D, et al. PALB2, which encodes a BRCA2-interacting protein, is a breast cancer susceptibility gene. *Nat Genet*. 2007;39:165–167.
8. Hartley T, Cavallone L, Sabbaghian N, Silva-Smith R, Hamel N, et al. Mutation analysis of PALB2 in BRCA1 and BRCA2-negative breast and/or ovarian cancer families from Eastern Ontario, Canada. *Hered Cancer Clin Pract*. 2014; 12(19):1–9.
9. Foulkes WD, Ghadirian P, Akbari MR, et al. Identification of a novel truncating PALB2 mutation and analysis of its contribution to early-onset breast cancer in French-Canadian women. *Breast Cancer Res*. 2007;9:R83.
10. Tischkowitz M, Xia B, Sabbaghian N, et al. Analysis of PALB2/FANCN associated breast cancer families. *Proc Natl Acad Sci USA*. 2007;104: 6788–6793.
11. Garcia MJ, Fernandez V, Osorio A, et al. Analysis of FANCB and FANCN/PALB2 fanconi anemia genes in BRCA1/2-negative Spanish breast cancer families. *Breast Cancer Res Treat.* 2009;113:545–551.
12. Cao AY, Huang J, Hu Z, et al. The prevalence of PALB2 germline mutations in BRCA1/BRCA2 negative Chinese women with early onset breast cancer or affected relatives. *Breast Cancer Res Treat.* 2009; 114:457–462.
13. Bogdanova N, Sokolenko AP, et al. PALB2 an and Russimutations in German patients with bilateral breast cancer. *Breast Cancer Res Treat*. 2011;126(2):545–550.
14. Casadei S, Norquist BM, Walsh T, et al. Contribution of inherited mutations in the BRCA2-interacting protein PALB2 to familial breast cancer. *Cancer Res*. 2011;71(6):2222–2229.
15. Nikkilä J, Parplys AC, Pylkäs K, et al. Heterozygous mutations in PALB2 cause DNA replication and damage response defects. *Nat Commun.* 2013;4(2578):1–8.
16. Antoniou AC, Casadei S, Heikkinen T, et al. Breast-cancer risk in families with mutations in PALB2. *N Engl J Med.* 2014;371(6):497–506.
17. Gunnarsson H, Arason A, Gillanders EM, et al. Evidence against PALB2 involvement in Icelandic breast cancer susceptibility. *J Negat Results Biomed*. 2008;7(5):1–4.
18. Poumpouridou N, Kroupis C. Hereditary breast cancer: Beyond BRCA genetic analysis; PALB2 emerges. *Clin Chem Lab Med*. 2011; 50(3):423–434.
19. Smith EC: PALB2 and the risks for cancer: Implications for clinical care. *Oncol Nurs Forum*. 2015;42:100–102.
20. Tischkowitz M, Capanu M, Sabbaghian N, et al. Rare germline mutations in PALB2 and breast cancer risk: A population-based study. *Hum Mutat.* 2012;33(4):674–680.
21. Janatova M, Kleibl Z, Stribrna J, et al. The PALB2 gene is a strong candidate for clinical testing in BRCA1- and BRCA2-negative hereditary breast cancer. *Cancer Epidemiol Biomarkers Prev.* 2013;22(12): 2323–2332.
22. Southey MC, Teo ZL, Dowty JG, et al. A PALB2 mutation associated with high risk of breast cancer. *Breast Cancer Research.* 2010; 12(R109):1–10.
23. Heikkinen T, Kärkkäinen H, Aaltonen K, et al. The breast cancer susceptibility mutation PALB2 1592delT is associated with an aggressive tumor phenotype. *Clin Cancer Res*. 2009; 15(9):3214–3222.
24. Fernandes PH, Saam J, Peterson J, et al. Comprehensive sequencing of PALB2 in patients with breast cancer suggests PALB2 mutations explain a subset of hereditary breast cancer. *Cancer.* 2014; 120(7):963–967.
25. Blanco A, de la Hoya M, Osorio A, et al. Analysis of PALB2 gene in BRCA1/BRCA2 negative Spanish hereditary breast/ovarian cancer families with pancreatic cancer cases. *PLoS One*. 2013; 8(7):e67538.
26. Stadler ZK, Salo-Mullen E, Sabbaghian N, et al. Germline PALB2 mutation analysis in breast-pancreas cancer families. *J Med Genet*. 2011; 48:523–525.
27. Adank MA, van Mil SE, Gille JJ, et al. PALB2 analysis in BRCA2-like families. *Breast Cancer Res Treat.* 2011;127:357–362.
28. Hofstatter EW, Domchek SM, Miron A, et al. PALB2 mutations in familial breast and pancreatic cancer. *Fam Cancer.* 2011;10:225–231.
29. Peterlongo P, Catucci I, Pasquini G, et al. PALB2 germline mutations in familial breast cancer cases with personal and family history of pancreatic cancer. *Breast Cancer Res Treat.* 2011;126:825–828.
30. Buisson R, Dion-Cote AM, Coulombe Y, et al. Cooperation of breast cancer proteins PALB2 and piccolo BRCA2 in stimulating homologous recombination. *Nat Struct Mol Biol.* 2010;17:1247–1254.

$P2Y_{12}$ receptor gene polymorphism and the risk of resistance to clopidogrel: A meta-analysis and review of the literature

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Abstract

A number of investigators have evaluated the association between T744C, G52T and C34T polymorphisms in the $P2Y_{12}$ receptor gene and clopidogrel resistance (CR), but the results of their research are controversial. To quantify the evidence addressing this issue, we performed a meta-analysis of all available studies to evaluate the above association between the 3 different $P2Y_{12}$ genotypes and CR in patients suffering from cardiovascular diseases. This study included articles up to October 2015. We performed a systematic search of PubMed, Embase, Web of Science, Cochrane database, China National Knowledge Infrastructure (CNKI) and WanFang database. Articles meeting the inclusion criteria were included and accumulated by meta-analysis including 5769 participants from 15 individual studies. For G52T polymorphism, a significant relationship between the $P2Y_{12}$ receptor gene and CR was found under the dominant genetic model ($p < 0.05$). There was a clear positive correlation between the C34T polymorphism and CR under the dominant, recessive, additive genetic models, respectively ($p < 0.05$). The evidence from the present meta-analysis indicates that $P2Y_{12}$ receptor gene C34T and G52T polymorphism might be a risk factor for the poor response to the platelet in patients on clopidogrel therapy, whereas a lack of association was found for T744C polymorphism examined by various genetic models.

Key words: polymorphism, cardiovascular diseases, resistance, clopidogrel, $P2Y_{12}$

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Increased platelet activation and thrombus formation are involved in the development and progression of cardiovascular diseases (CVDs).[1] Current oral anti-platelet agent thienopyridine clopidogrel inhibits adenosine diphosphate (ADP) binding to platelet ADP receptor $P2Y_{12}$ on the platelet surface, and thus inhibits platelet aggregation. $P2Y_{12}$ has been shown to trigger platelet activation when stimulated in this receptor. Clopidogrel is effective in decreasing platelet activation and the subsequent risk of atherosclerosis-related CVDs including myocardial infraction, coronary heart disease and ischemic stroke.[2] However, in recent years, the concept of clopidogrel resistance (CR) or poor responsiveness, is increasingly evoked in the cardiac literature.[3]

The mechanisms of CR have not been fully characterized but are likely to be multifactorial, involving possible genetic polymorphisms, drug interaction and variable absorption or metabolism.[4] Five polymorphisms (T744C, C34T G52T, ins801A and C139T) of the $P2Y_{12}$ gene have been identified by Fontana et al.[5] It has been proposed that genetic polymorphisms of the platelet surface receptor affect the responsiveness to clopidogrel. Some have suggested that polymorphisms of T744C and C34T G52T contribute to CR.[6–8] However, this proposal is controversial, because other studies have shown that the correlations between the mutational statuses and poor response to clopidogrel therapy were not statistically significant.[7,9] Thus, in the present study, a meta-analysis including 5769 patients with CVDs was performed to clarify whether or not clopidogrel response to the platelet may be affected by $P2Y_{12}$ receptor gene polymorphism in patients with various types of CVDs. This study may help to identify the correct therapeutic approach for each individual patient with CVDs in order to maximize the therapeutic effect.

Methods

Search strategies

Published studies on the association between $P2Y_{12}$ receptor gene polymorphism and CR were retrieved by searching the following English and Chinese bibliographic databases: PubMed, Embase, Web of Science, Cochrane database, China National Knowledge Infrastructure (CNKI) and WanFang database. The search strategy was based on the following keywords: "$P2Y_{12}$" or "T744C" or "C34T" or "G52T" and "polymorphism" or "mutation" or "genotype" or "allele" and "clopidogrel" and "resistance" or "response". The literature search was updated to October 2015.

Study selection

Studies included in the current meta-analysis had to be consistent with these criteria: a) the association of $P2Y_{12}$ gene polymorphism and CR was included; b) provides the genotype frequency in the target population; c) was a case-control study; d) all patients had received clopidogrel therapy. Studies were excluded if they were: (1) duplicates of previous publications; (2) based on incomplete data; (3) meta-analyses, letters, reviews, or editorial articles; (4) publications with non-English abstracts. If more than 1 study by the same author using the same case series was published, only the study with the largest sample size or the most recent publication was included.

Data extraction

Two reviewers independently extracted the information, including the name of first author, year of publication, country, disease name, diagnostic standard of CR and genotypes and the frequency in patients with and without CR. In cases of conflicting evaluations, disagreements were resolved through discussions among all of the 5 authors.

Statistical analysis

The strength of the association between $P2Y_{12}$ gene polymorphism and CR was represented by odds ratios (OR) and 95% confidence intervals (CI). The OR and 95% CI were calculated according to 3 genetic models of inheritance: dominant (heterozygotes + homozygotes vs wild-type homozygotes), recessive (wild-type homozygotes + heterozygotes vs homozygotes), and additive (heterozygotes + homozygotes vs wild-type homozygotes + + heterozygotes). Heterogeneity between the results of different studies was examined using a χ^2 test. A 2-side value of $p < 0.05$ was considered statistically significant as previously described.[10] A fixed effects model was used when $p < 0.05$, and a random effects was used when $p > 0.05$. All analyses were performed by RevMan, v. 5.2 for Windows (Cochrane Collaboration, Oxford, UK) using our previously described method.[11]

Results

Study selection and characteristics

As shown in Fig. 1, 297 relevant studies were identified using the key words by a computerized search of PubMed, Embase, Web of Science, Cochrane database, CNKI and WanFang database. According to the selection criteria as described in methods, 14 studies were included for meta-analysis. Of the studies, there were 9 comparisons for T744C polymorphism, 7 and 6 comparisons for G52T and C34T, respectively.[6,8,9,12–22] The studies were conducted in France, the United States, China, Croatia, Egypt, and the Czech Republic. The characteristics of the studies included in this meta-analysis are presented in Table 1.

Table 1. Characteristics of the studies included in the meta-analysis

Position	First author	Year	Country	Disease	Dose (mg)	Definition of CR	CR		NCR	
T744C							CC + CT	TT	CC + CT	TT
	Cuisset[13]	2007	France	ACS	600	HPPR = ADP-induced aggregation > 70%	106	347	32	112
	Lev[14]	2007	USA	PAD	300	percent inhibition of ADP ≤ 10%	6	23	27	64
	Wang[18]	2009	China	CHD	300	percent inhibition of ADP ≤ 10%	36	57	67	87
	Sun[17]	2011	China	CAD	300	HPPR = ADP-induced aggregation > 70%	53	93	178	291
	Galic[6]	2013	Croatia	CHD	300	percent inhibition of ADP ≤ 10%	3	11	13	23
	Zoheir[8]	2013	Egypt	ACS	NA	HPPR = ADP-induced aggregation > 70%	1	23	10	6
	Shi[16]	2013	China	cerebral infarction	300	percent inhibition of ADP ≤ 10%	7	5	22	69
	Chen[12]	2014	China	ischemic stroke	75	percent inhibition of ADP ≤ 10%	5	26	25	40
	Shi[15]	2014	China	cerebral infarction	75	percent inhibition of ADP ≤ 10%	16	20	27	58
G52T							TT + GT	GG	TT + GT	GG
	Wang[18]	2009	China	CHD	300	percent inhibition of ADP ≤ 10%	41	50	53	96
	Bonello[19]	2010	France	CAD	600	percent inhibition of ADP ≤ 10%	0	10	6	27
	Sun[17]	2011	China	CAD	300	percent inhibition of ADP ≤ 10%	37	109	95	374
	Liu[9]	2011	China	CHD	300	percent inhibition of ADP ≤ 10%	9	26	13	61
	Li[20]	2014	China	CAHD	75	percent inhibition of ADP ≤ 10%	52	48	100	170
	Chen[12]	2014	China	ischemic stroke	75	percent inhibition of ADP ≤ 10%	4	28	17	47
	Zhao[21]	2015	China	ACS	75	HPPR = ADP-induced aggregation > 70%	10	16	10	48
C34T							TT + CT	CC	TT + CT	CC
	Wang[18]	2009	China	CHD	300	percent inhibition of ADP ≤ 10%	58	35	56	98
	Sun[17]	2011	China	CAD	600	percent inhibition of ADP ≤ 10%	91	55	230	239
	Galic[6]	2013	Croatia	CHD	300	percent inhibition of ADP ≤ 10%	8	6	18	18
	Tang[22]	2013	China	ACS	300	percent inhibition of ADP < 30%	53	55	167	302
	Li[20]	2014	China	CAHD	75	percent inhibition of ADP ≤ 10%	74	26	84	186
	Zhao[21]	2015	China	ACS	75	HPPR = ADP-induced aggregation > 70%	12	14	19	39

CR – clopidogrel resistance; NCR – non-clopidogrel resistance; ACS – acute coronary syndrome; HPPR – high post-treatment platelet reactivity; PAD – peripheral arterial disease; CHD – coronary heart disease; CAD – coronary artery disease; AMI – acute myocardial infarction; NA – not applicable; CAHD – coronary atherosclerotic heart disease.

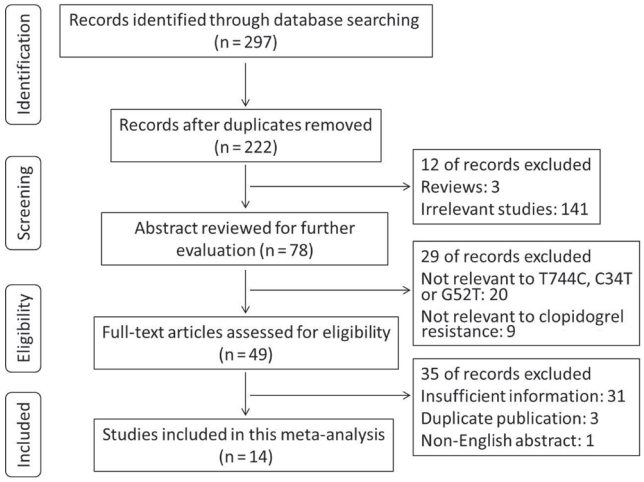

Fig. 1. Flow diagram of study selection procedure

Quantitative data synthesis

The conclusions based on the included studies showed that: (1) $P2Y_{12}$ receptor gene T744C polymorphism had no association with CR (OR: 0.88, 95% CI: 0.58–1.33, p = 0.54), (2) CVD cases with CR had a significantly higher frequency of CT and TT genotypes (OR: 1.45, 95% CI: 1.14–1.85, p = 0.003) than the CC (wild type) genotype of G52T under the dominant genetic model, (3) an association between $P2Y_{12}$ C34T polymorphism and CR was detected under the recessive (OR: 2.19, 95% CI: 1.44–3.34, p = 0.0003), dominant (OR: 2.30, 95% CI: 1.50–3.51, p = 0.0001) and additive (OR: 0.57, 95% CI: 0.47–0.71, p < 0.00001) genetic models. These results revealed that mutant genotypes (G52T/C34T) of the $P2Y_{12}$ receptor gene might be associated with an increased risk of CR.

Subgroup analysis and sensitivity analysis

For T744C and C34T polymorphisms, high heterogeneity was observed (I^2 = 63%) according to the reported quantifying heterogeneity approach.[10] Subgroup analysis of the T744C genetic polymorphism was performed to determine the potential sources of the heterogeneity (Table 2). We classified the studies based on the geographic region (European and American, Asian). In the results, whether geographic region was adjusted or not, this association did not change (p > 0.05). For the G52T polymorphism, we did not perform a subgroup analysis due to the limited number of included studies (only 2 studies included from European and American regions).

We removed one study by Galic et al. in the review due to T744C genotype distribution in the control groups of these studies deviating from the Hardy-Weinberg equilibrium (HWE) and found that the association (OR= 1.17, 95% CI: 0.86–1.60, p = 0.32) was not significantly altered after exclusion of the study, indicating that the result of the meta-analysis was stable.[6] In addition, potential publication bias or heterogeneity was detected using visual as-

Table 2. Subgroup analyses of the association between $P2Y_{12}$ receptor gene T744C polymorphism and CR according to region

Subgroup	Pooled OR (95% CI)	z-value	p-value	Study number	$P_{heterogeneity}$ (I^2%)
European and American participants	0.93 (0.62–1.37)	0.38	0.70	3	0.41 (0%)
Asian participants	1.22 (0.98–1.53)	1.75	0.08	6	0.04 (57%)

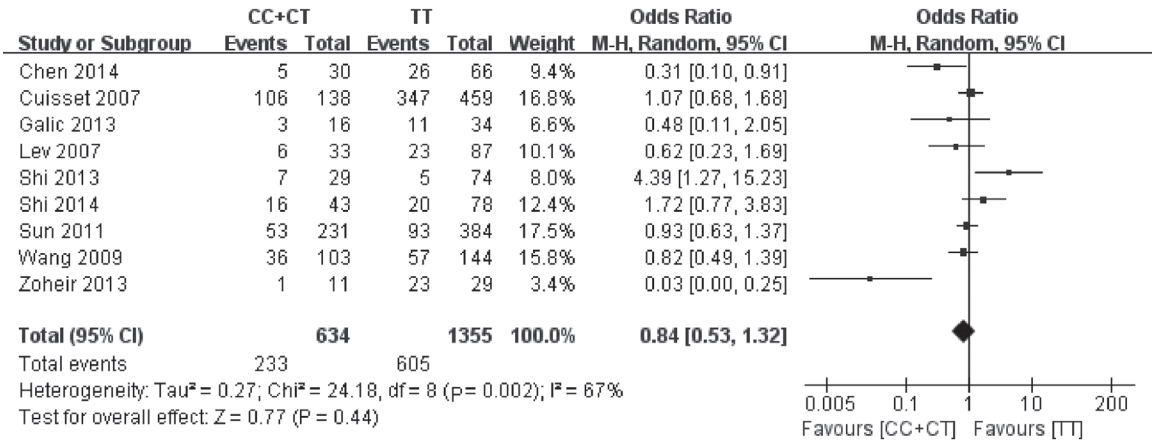

Fig. 2. Forest plot of OR of CR associated with $P2Y_{12}$ receptor gene T744C polymorphism in the dominant genetic model. Comparison: CC + CT vs TT

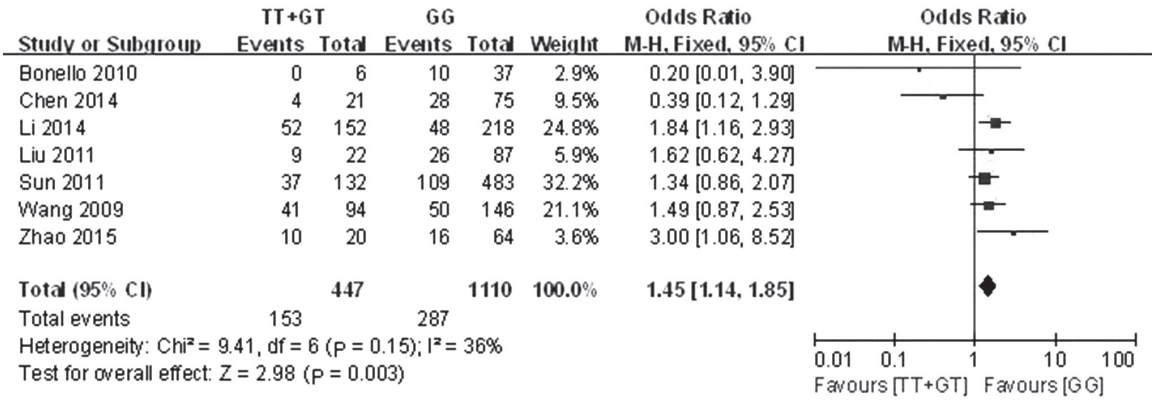

Fig. 3. Forest plot of OR of CR associated with $P2Y_{12}$ receptor gene G52T polymorphism in the dominant genetic model. Comparison: TT + GT vs GG

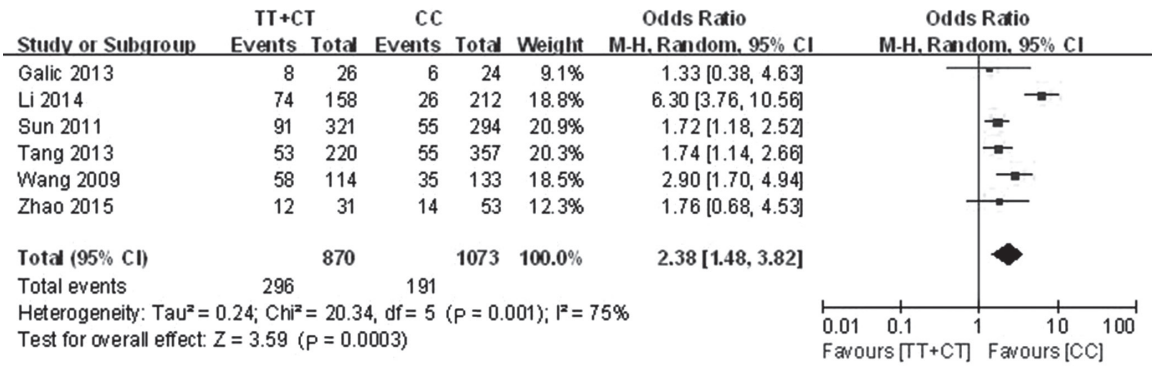

Fig. 4. Forest plot of OR of CR associated with $P2Y_{12}$ receptor gene C34T polymorphism in the dominant genetic model. Comparison: TT + CT vs CC

sessment of the Begg's funnel plot calculated by RevMan analyses. Funnel plots (Fig. 5) display symmetrical distribution of OR estimations, suggesting no publication bias.

Discussion

The strength of our study is the statistically most extensive studies to date addressing whether the risk of CR is associated with the frequencies of $P2Y_{12}$ receptor gene T744C, C34T and G52T genotypes in patients with CVDs. In the current review, 15 independent studies were included, comprised of 1829 patients with CR and 3940 patients without CR. For the first time, we used meta-analysis to summarize that there was probably a significant association between $P2Y_{12}$ receptor gene G52T/C34T polymorphism and CR.

The $P2Y_{12}$ receptor, activated by ADP, plays a critical

Table 3. Summary of meta-analysis of association of $P2Y_{12}$ receptor gene polymorphism and CR using recessive genetic and additive models

Model	Pooled OR (95% CI)	z-value	p- value	Study number	$P_{heterogeneity}$ (I^2%)
Recessive genetic model					
T744C (CC vs TT + TC)	1.08 (0.57–2.05)	0.23	0.82	4	0.13 (48%)
G52T (TT vs GG + GT)	2.32 (0.58–9.28)	1.18	0.24	3	0.48 (0%)
C34T (TT vs CC + CT)	2.19 (1.44–3.34)	3.64	0.0003*	4	0.20 (36%)
Additive genetic model					
T744C (T vs C)	1.01 (0.83–1.24)	0.12	0.90	5	0.49 (0%)
G52T (G vs T)	0.78 (0.58–1.04)	1.67	0.09	3	0.41 (0%)
C34T (C vs T)	0.57 (0.47–0.71)	5.29	< 0.00001*	4	0.14 (45%)

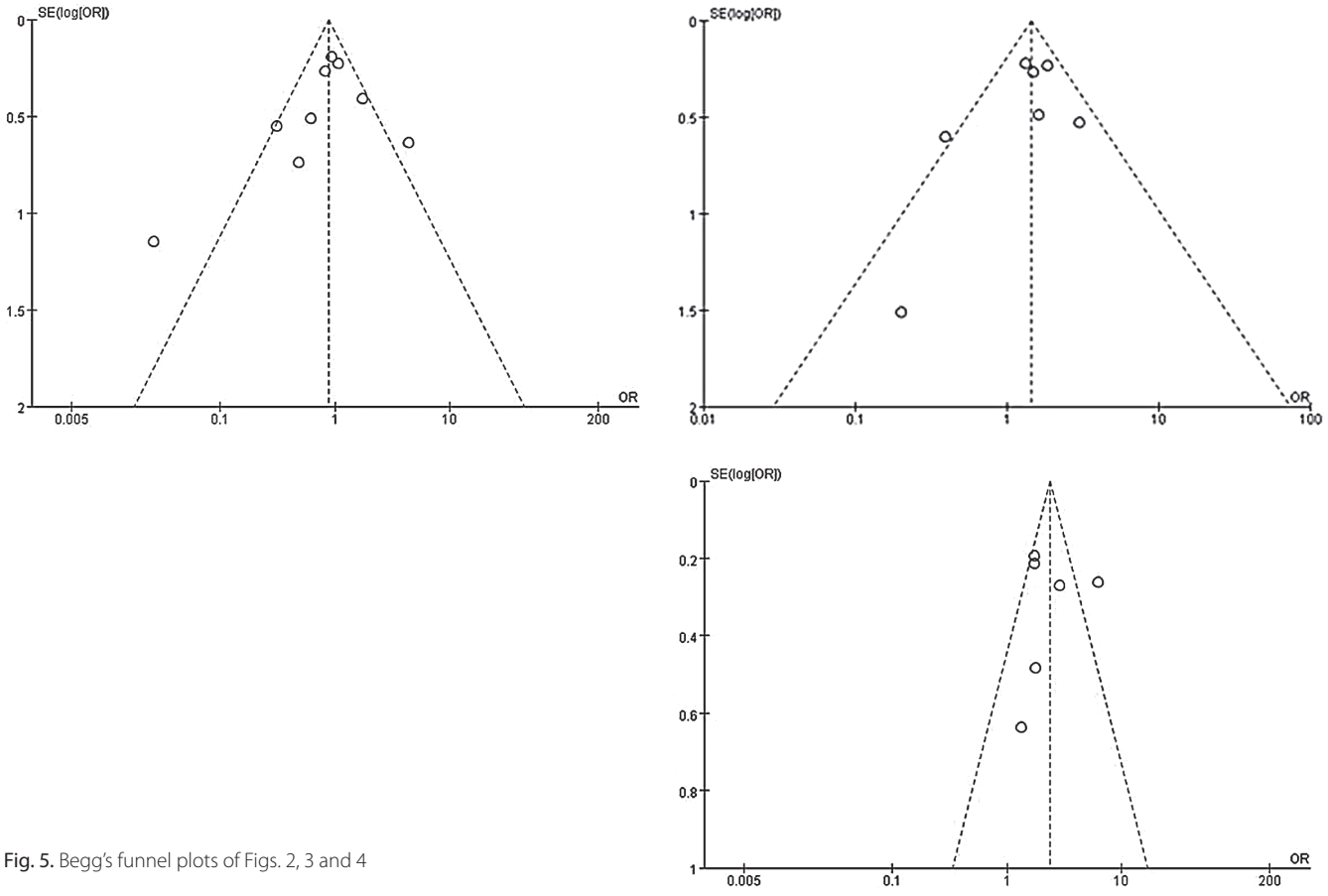

Fig. 5. Begg's funnel plots of Figs. 2, 3 and 4

role in platelet aggregation and is a target of antiplatelet therapeutic agents that has proven therapeutic value.[23] One of the first successful drugs is clopidogrel. Clopidogrel is a prodrug and its metabolite inhibits ADP receptor-mediated platelet aggregation. Clopidogrel is a milestone in the development of antiplatelet therapy and entails a reduction in the risk of atherothrombotic event, which is the leading cause of CVDs.[24,25] However, the concept of CR or poor responsiveness is increasing evoked in the cardiac literature in antiplatelet response.[3] Although numerous association studies reflecting the influence of $P2Y_{12}$ receptor gene polymorphisms on CR have been published, the results are controversial, possibly because of variations in heterogeneous population, sample sizes and other issues. A meta-analysis by systematically combining all the results of individual studies increases the power to detect an association.[26]

In the current meta-analysis, we did not find a significant association between the T744C polymorphism and CR in various genetic models. This finding suggests that the T744C polymorphism may be not susceptible to CR in patients with CVDs. In contrast, we also found that patients with a higher frequency of homozygous and heterozygous (TT + GT) genotypes of G52T had a higher risk of CR than wild-type (GG) genotypes (p = 0.003). For C34T polymorphism, despite the statistical heterogeneity of the studies associating $P2Y_{12}$ receptor gene C34T polymorphism under the dominant genetic model (p = 0.002,

I^2 = 71%), all of the included studies suggested a positive association between the risk of the CR and C34T polymorphism. We pooled these studies with a random-effects model and the result was consistent with the individual study (OR: 2.30, 95% CI: 1.50–3.51, p = 0.0001).

Variability in the definitions of CR and different regions of population possibly contributed to the heterogeneity in the comparisons. The use of antiplatelet therapy, including differing doses and lengths of the treatment, may also result in the heterogeneity. Antiplatelet drugs could influence the association of the $P2Y_{12}$ gene polymorphisms with clopidogrel resistance as this might affect clopidogrel and ADP binding.[27] In addition, an association was also found for the C34T gene polymorphism determined under the dominant, additive, and genetic models ($p < 0.05$). Conversely, in a previous study excluded in this meta-analysis, it was shown that there is lack of association between the frequency of C34T polymorphism and the antiplatelet aggregation response of clopidogrel.[28] Our results clearly indicate augmented CR for C34T polymorphism.

Although considerable effects have been found in the current study, there are some inherent limitations. First, the sample size is still relatively small in the current meta-analysis. Although 5489 total participants from 14 individual studies were included in the analysis, < 1600 participants were analyzed in the G52T polymorphism group. Fewer case subjects were included in some studies. Of the included studies, 3 had less than 100 participants.[6,8,19] Second, 3 different definitions of CR were adopted by researchers since a standard definition of CR was not available. This may slightly weaken the comparability of the data. Third, the overall ORs were calculated according to the unadjusted estimates. It has been reported that drug-drug interaction or coexisting polymorphisms of $P2Y_{12}$ and other genes may influence CR.[22,29] This may exist in the included studies and could interfere with the meta-analysis. Fourth, different clopidogrel doses (75–600 mg) and various times after initial therapy (6 h–7 days) were used in the included studies. Those may be a confounder in the analysis of the platelet aggregation test. Such confounding factors could not be extracted from the included studies due to a lack of information, but they limit the potential to draw robust conclusions.

In summary, this is the first meta-analysis to provide evidence that $P2Y_{12}$ G52T/C34T polymorphism is related to a poor response of clopidogrel in patients, reflected by platelet function assay. In contrast, a lack of association between T744C polymorphism and CR was found. This finding warrants further studies of larger sample sizes and multiple countries for a more sensitivity analysis. Furthermore, this study raises concerns about future studies addressing individual alternative clopidogrel-based antiplatelet therapy for patients with $P2Y_{12}$ C34T and/or G52T polymorphism, and encourages a precision medicine approach to the treatment of CVDs.

References

1. Hansson GK, Klareskog L. Pulling down the plug on atherosclerosis: Cooling down the inflammasome. *Nat Med.* 2011;17(7):790–791.
2. Behan MW, Storey RF. Antiplatelet therapy in cardiovascular disease. *Postgrad Med J.* 2004;80(941):155–164.
3. Hurst NL, Nooney VB, Raman B, et al. Clopidogrel "resistance": Pre- vs post-receptor determinants. *Vascul Pharmacol.* 2013;59(5–6):152–161.
4. Siller-Matula J, Schror K, Wojta J, et al. Thienopyridines in cardiovascular disease: Focus on clopidogrel resistance. *Thromb Haemost.* 2007;97(3):385–393.
5. Fontana P, Dupont A, Gandrille S, et al. Adenosine diphosphate-induced platelet aggregation is associated with P2Y12 gene sequence variations in healthy subjects. *Circulation.* 2003;108(8):989–995.
6. Galic E, Vrbanic L, Kapitanovic S, et al. P2RY12 gene polymorphisms and effect of clopidogrel on platelet aggregation. *Collegium antropologicum.* 2013;37(2):491–498.
7. Staritz P, Kurz K, Stoll M, et al. Platelet reactivity and clopidogrel resistance are associated with the H2 haplotype of the P2Y12-ADP receptor gene. *Int J Cardiol.* 2009;133(3):341–345.
8. Zoheir N, Abd Elhamid S, Abulata N, et al. P2Y12 receptor gene polymorphism and antiplatelet effect of clopidogrel in patients with coronary artery disease after coronary stenting. *Blood Coagul Fibrinolysis.* 2013;24(5):525–531.
9. Liu YM, Liu NF. Evaluating association of P2Y12 gene polymorphisms with clopidogrel response variability in Chinese patients undergoing PCI. *Journal of Southeast University.* 2011;30(3):449–453.
10. Higgins JP, Thompson SG, Deeks JJ, et al. Measuring inconsistency in meta-analyses. *BMJ.* 2003;327(7414):557–560.
11. Zhang SY, Chan SW, Zhou X, et al. Meta-analysis of association between ALDH2 rs671 polymorphism and essential hypertension in Asian populations. *Herz.* 2015;40:203–208.
12. Chen XM, Jin J, Huang M, et al. Association between CYP2C19, P2Y12 genetic polymorphisms and clopidogrel resistance in ischemic stroke patients. *China Pharmaci.* 2014;17(6):885–888.
13. Cuisset T, Frere C, Quilici J, et al. Role of the T744C polymorphism of the P2Y12 gene on platelet response to a 600-mg loading dose of clopidogrel in 597 patients with non-ST-segment elevation acute coronary syndrome. *Thromb Res.* 2007;120(6):893–899.
14. Lev EI, Patel RT, Guthikonda S, et al. Genetic polymorphisms of the platelet receptors P2Y(12), P2Y(1) and GP IIIa and response to aspirin and clopidogrel. *Thromb Res.* 2007;119(3):355–360.
15. Shi HT, Zhou BR, Wang R, et al. Analysis of clinical characteristics of P2Y12 and CYP2C19 mutation genes and their relationship with the incidence of dynamic clopidogrel resistance in patients after cerebral infarction. *J Apoplexy and Nervous Diseases.* 2014;31(10):9–13.
16. Shi ST, Li YJ, Zhang MJ, et al. Related factors of clopidogrel resistance in Han patients with cerebral infarction. *J Shanxi Med Univ.* 2013;44(12):953–956.
17. Sun Y, Han YL, Zhang XL, et al. Association of T744C polymorphism of the P2Y12 gene with clopidogrel resistance in North Chinese patients of coronary artery disease. *China Medical Engineering.* 2011;19(04):44–47.
18. Wang JJ. Influences of P2Y12 (C34T, G52T, iT744C). Polymorphisms on clopidogrel resistance in patients with coronary heart disease. *Fujian Medical University.* 2009:1–51.
19. Bonello L, Bonello-Palot N, Armero S, et al. Impact of P2Y12-ADP receptor polymorphism on the efficacy of clopidogrel dose-adjustment according to platelet reactivity monitoring in coronary artery disease patients. *Thromb Res.* 2010;125(4):167–170.
20. Li XJ, Chen XM. Association between clopidogrel resistance and polymorphism of platelet adenosine diphosphate receptor in patients with coronary atherosclerotic disease. *Journal of Zhejiang University-Science B.* 2014;43(3):333–338.
21. Zhao M, Chen BX, Du FH. Association between P2Y12 gene C34T and G52T platelet receptor polymorphisms and clopidogrel efficacy. *Lab Med Clin.* 2015;12(5).
22. Tang XF, Zhang JH, Wang J, et al. Effects of coexisting polymorphisms of CYP2C19 and P2Y12 on clopidogrel responsiveness and clinical outcome in patients with acute coronary syndromes undergoing stent-based coronary intervention. *Chin Med J (Engl).* 2013;126(6):1069–1075.

23. Dorsam RT, Kunapuli SP. Central role of the P2Y12 receptor in platelet activation. *J Clin Invest*. 2004;113(3):340–345.
24. Bhatt DL, Fox KA, Hacke W, et al. Clopidogrel and aspirin versus aspirin alone for the prevention of atherothrombotic events. *N Engl J Med*. 2006;354(16):1706–1717.
25. Viles-Gonzalez JF, Fuster V, Badimon JJ. Atherothrombosis: A widespread disease with unpredictable and life-threatening consequences. *Eur Heart J*. 2004;25(14):1197–1207.
26. Ioannidis JP, Ntzani EE, Trikalinos TA, et al. Replication validity of genetic association studies. *Nat Genet*. 2001;29(3):306–309.
27. Angiolillo DJ, Fernandez-Ortiz A, Bernardo E, et al. Variability in individual responsiveness to clopidogrel: Clinical implications, management, and future perspectives. *J Am Coll Cardiol*. 2007;49(14):1505–1516.
28. Chen BL, Li Q, Xie W, et al. P2Y12 gene polymorphism in Chinese healthy population and patients with coronary artery disease and the association with effect of clopidogrel. *Chinese Journal of New Drugs*. 2008;17(16):1435–1439.
29. Lau WC, Waskell LA, Watkins PB, et al. Atorvastatin reduces the ability of clopidogrel to inhibit platelet aggregation: A new drug-drug interaction. *Circulation*. 2003;107(1):32–37.

Antiarrhythmic properties of atrial pacing

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Abstract

Bradycardia, atrial stretch and dilatation, autonomic nervous system disorders, and the presence of triggers such as atrial premature contractions, are factors which predispose a person to paroxysmal AF. Atrial pacing not only eliminates bradycardia but also prevents atrial premature contractions and dispersion of refractoriness, which are a substrate for atrial fibrillation. As the prolonged duration of atrial activation during pacing, especially from locations changing the physiological pattern of this activation (right atrium lateral wall, right atrium appendage), negatively influences both a mechanical and an electrical function of the atria, the atrial pacing site affects an atrial arrhythmogenesis. A conventional atrial lead location in the right atrium appendage causes non-physiological activation propagation, resulting in a prolongation of the activation time of both atria. This location is optimal according to a passive fixation of the atrial lead but the available contemporary active fixation leads could potentially be located in any area of the atrium. There is growing evidence of the benefit of pacing, imitating the physiological propagation of impulses within the atria. It seems that the Bachmann's bundle pacing is the best pacing site within the atria, not only positively influencing the atrial mechanical function but also best fulfilling the so-called atrial resynchronization function, in particular in patients with interatrial conduction delay. It can be effectively achieved using only one atrial electrode, and the slight shortening of atrioventricular conduction provides an additional benefit of this atrial pacing site.

Key words: atrial pacing, alternative sites, atrial fibrillation, Bachmann's bundle

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In the electrical conduction system of the heart, an impulse is generated by the sinoatrial node, located between the superior vena cava ostium and the crista terminalis, and it is propagated down and ahead through the right atrium and simultaneously to the left atrium.[1] Electrical coupling of both atria is predominantly provided by connections at the level of the Bachmann's bundle and the coronary sinus. The true septum (the fossa ovalis and its limbus) is asynchronous and discordant, usually without contralateral conduction during the sinus rhythm or the atrial pacing.[2] This course of activation leads to a proper propagation – the 1st part to contract is the right atrium free wall, then the interatrial septum and afterwards the posterior, anterior and lateral walls of the left atrium. An efficient interatrial conduction leads to a physiological delay of the left atrium contraction/interatrial delay which is 24 ± 21 ms. Thereby, the activation of both atria is represented by a single P-wave in the surface ECG.[2]

Pathophysiology of atrial arrhythmias

The pathophysiological mechanisms underlying atrial arrhythmia are not yet explicitly understood. It is probably triggered by premature atrial activations, most commonly localized or coming from the region of pulmonary veins or the superior vena cava, and maintained by inhomogeneous atrial conduction velocities and refractory periods. Atrial premature beats can trigger atrial fibrillation in at least 3 ways: an atrial premature contraction after a pause in the sinus rhythm, atrial premature beats with a short coupling interval (the P-on-T phenomenon), and short-long-short atrial sequences. Betts et al. mapped the left atrial endocardial activation during the sinus rhythm and coronary sinus pacing in patients with paroxysmal atrial fibrillation and discovered areas of functional block in the posterior left atrium wall in proximity to the pulmonary veins and suggested that they can serve as a substrate for reentrant arrhythmias, including an atrial flutter and atrial fibrillation.[3] Bradycardia, atrial dilatation, disturbances of the autonomic nervous system, atrial stretch, and the presence of triggers such as atrial premature contractions are factors which predispose a person to paroxysmal AF.

Atrial fibrillation constitutes the most common arrhythmia in the general population and its incidence doubles with each successive age decade over 50 years, from 0.5% at age 50–59 years to almost 9% at age 80–89 years. An interesting epidemiological study on atrial fibrillation was conducted by O'Neal et al.[4] A relatively large group of participants (5226) aged 65 years or over were observed for atrial fibrillation development. The analysis showed that a low heart rate (below 60 beats/min) was associated with an increased risk of this arrhythmia, while no such correlation was observed for a heart rate above 90 beats/min. This could suggest underlying autonomic abnormalities or subclinical sinus node dysfunction.

Atrial fibrillation is associated with atrial remodeling, which may be both the substrate and the consequence of the arrhythmia. Different kinds of remodeling have been described, such as an autonomic neural remodeling, or an electrical and a structural remodeling. Uhm et al. compared effective refractory periods (ERP) in 648 patients with AF and drew the conclusion that patients with persistent AF have shorter ERP compared to those with paroxysmal arrhythmia, probably due to an electrical remodeling of the atria. On the other hand, patients with structural heart disease and sinus node dysfunction have longer ERP than patients with 'lone' AF which is associated with a structural remodeling, ventricular diastolic dysfunction or left atrium enlargement.[5]

The influence of atrial pacing

As atrial fibrillation is an arrhythmia paroxysmal in nature, the proper assessment of the antiarrhythmic influence of any kind of procedure, device or pharmacological treatment is difficult. Because of a higher prevalence of atrial fibrillation in patients with pacemakers and still an insufficient efficacy of pharmacotherapy in this arrhythmia, the best pacing mode and the best lead location is still being discussed. As the prolonged duration of the atrial activation during pacing, especially from locations changing the physiological pattern of this activation (right atrium lateral wall, right atrium appendage), has a negative impact on both a mechanical and an electrical function of the atria, the impact of atrial pacing site locations on atrial arrhythmogenesis can be observed. So far the study findings on the best pacing site are inconsistent.

The pacing of the atria can eliminate AF triggers such as premature atrial activations and prevent changes in the refractoriness they cause, supporting the atrial synchrony. Atrial pacing not only eliminates bradycardia but also prevents atrial premature contractions and a dispersion of refractoriness which are a substrate for atrial fibrillation. A premature atrial contraction, especially during the vulnerable period of refractoriness, negatively influences atrial myocardium conduction velocity. In a study by Hoffmann et al., 46% of atrial fibrillation episodes were preceded by atrial premature contractions and 39% by bradycardia.[6]

The superiority of atrial pacing vs ventricular pacing

It is now well established that atrial pacing is superior to ventricular pacing for the prevention of atrial fibrillation as it prevents atrial premature beats and atrial bradycardia episodes and helps to avoid an increase of atrial

pressure associated with atrioventricular dyssynchrony. It is well documented that single lead ventricular pacing, which disrupts the normal atrioventricular synchrony, is associated with a higher incidence of atrial fibrillation than dual chamber pacing (DDD) preserving atrioventricular synchrony. In a study by Andersen et al., patients with sick sinus syndrome (SSS) were observed for 8 years. Atrial pacing was associated with less atrial fibrillation and thromboembolism, higher survival and less heart failure compared to ventricular pacing.[7] A lower incidence of atrial fibrillation during atrial pacing was also shown in a large group of 1474 patients in the Canadian Trial of Physiological Pacing, however it did not affect the risk of stroke or cardiovascular death.[8]

The influence of atrial pacing on atrial arrhythmogenesis

Hjortshøj et al. proved in a group of 396 patients that there is no association between the percentage of atrial pacing and the development of AF in patients with sick sinus syndrome, although earlier studies had given different results on this matter.[9] Elkayam et al. demonstrated that both atrial and ventricular pacing in patients with sick sinus syndrome are associated with a higher risk of AF. A group of 1507 patients was divided into quartiles on the basis of the atrial pacing percentage: The higher this percentage was, the higher the incidence of AF.[10] On the other hand, in a study by Inoue et al. in patients with the atrial pacing percentage (AP%) < 50%, the AF burden was greater than in those with AP% > 50%, which would suggest a protective influence of atrial pacing on atrial fibrillation, however the effect was not always immediate.[11]

According to the results of the DANPACE study, a dual-chamber DDD(R) pacing with preserved atrioventricular conduction in SSS should be preferred over a single-lead AAIR pacing as the latter is associated with a two-folded risk of reoperation and a higher risk of paroxysmal AF. There was, however, no difference in the overall mortality, chronic atrial fibrillation, heart failure and stroke between those 2 modes of pacing.[12] Such results are consistent with those of Kuniewicz et al., who also pointed out that tachycardia-bradycardia syndrome and a lack of antiarrhythmic pharmacological therapy with beta-blockers and amiodarone increases the risk of a persistent AF.[13]

An analysis of the data from the DANPACE study showed that AF burden is associated with a longer baseline PQ interval, however neither with the percentage of ventricular pacing nor with the length of programmed AV interval.[14] Similarly in the MinVPace study, although some algorithms reduced the percentage of ventricular pacing, there was no reduction of atrial fibrillation incidence.[15] These 2 results can suggest that a longer PQ interval or a greater percentage of ventricular pacing are only indicators of a larger scope of conduction abnormalities present also in the atrial muscle, leading to atrial fibrillation.

As already mentioned, different pacing sites vary in electrophysiological properties. A conventional atrial lead location in the right atrium appendage causes a non-physiological activation spreading, resulting in a prolongation of atria activation which can be seen as a broadened P-wave in the surface electrocardiogram.

Bennett used the intracardiac electrophysiological study to assess atrial activation parameters during stimulation at the right atrial appendage, atrial septum, coronary sinus ostium and during a simultaneous stimulation of the right atrial appendage and the ostium of coronary sinus. The atrial activation time was longer during the right atrium appendage pacing compared to other locations. Atrial septum, ostium of coronary sinus and dual site stimulation showed no significant differences. This study suggests potential benefits from intraatrial septum pacing as shortening the activation time of both atria diminishes the dispersion of refractoriness. Dual site pacing showed no additional benefits, which suggests that the septal pacing should be preferred because of the simplicity of the procedure.[16]

Katsivas et al. implanted an atrial lead in the interatrial septum in patients with drug refractory paroxysmal atrial fibrillation and broad P-waves in the surface ECG and proved that this location resulted in shortening P-waves from 118 ± 10 ms to 93 ± 7 ms.[17] The interatrial conduction time during pacing was shortened in comparison to sinus rhythm (115 ± 18.9 ms vs 97.8 ± 10.3 ms).

The manifestation of the atrial activation is a P-wave in the surface electrocardiogram. Lengthening its duration is an easily-measured manifestation of a prolonged atrial activation and is associated with intra- and interatrial conduction disturbances. Prolongation of the P-wave duration is associated with the development of atrial tachyarrhythmias in patients with a pacemaker.[18] Kirstensen et al. conducted a study in which they assessed atrial parameters in patients with sick sinus syndrome during sinus rhythm and, respectively, 70 and 100 bpm atrial pacing with the lead in the interatrial septum or high right atrium. High right atrium pacing resulted in longer P-waves than pacing with septal location, but during high rate pacing the P-wave was significantly longer than during pacing at 70 bpm in both lead locations. The conclusion was that neither P-wave dispersion nor its duration were predictors of atrial fibrillation paroxysm.[19] De Sisti et al., however, proved that in patients with sick sinus syndrome after permanent pacemaker implantation, the prolonged P-wave is an independent risk factor of atrial fibrillation.[20]

Advanced interatrial block, presenting in the surface ECG as a P-wave duration over 120 ms with biphasic +/- P-waves in lead II, III and aVF as an expression of a retrograde left atrium activation, is associated with an increased risk of any atrial tachyarrhythmia.[21] De Sisti et al.

studied the sinus P-wave morphology, evaluated in leads II and III, in 140 patients with a recurrent paroxysmal AF who received pacemakers. A multivariate analysis showed that drugs before pacing and P-wave duration were independent predictors of AF, and an abnormal P-wave (biphasic +/– or notched +/+) was the most important predictor of a permanent AF.[21] Endoh et al. investigated 57 patients with SSS with an atrial lead implanted in the right atrium appendage, who were divided into 2 groups: 23 patients without AF before pacing and 34 with AF before pacing. The latter group was further divided into 2 subgroups: 20 patients with a paced P-wave duration over 130 ms in leads II and V1, and the remaining 14 patients. The sinus P-wave was significantly longer in the group with a history of AF whilst a recurrence of the arrhythmia was significantly higher in the group with both a history of AF and a longer paced P-wave. This study suggests that a coincidence of SSS with AF is associated with intra-atrial conduction disturbances which may be aggravated by RAA pacing, especially in patients with a prolonged paced P-wave in whom other pacing modalities may be needed to shorten the paced P-wave.[22]

Atrial electrode position

Stabile et al. studied 47 patients with paroxysmal atrial fibrillation requiring demand pacing who underwent dual chamber pacemaker implantation and an electrophysiological study.[23] They proved that single site atrial pacing is effective in reducing AF recurrences, however an atrial conduction delay and refractoriness dispersion decrease its efficacy. Pacing at RA sites at which a preferential interatrial conduction exists may pre-excite the left atrium and shorten the total atrial activation time which may result in a reduction of the susceptibility to AF.[22]

Interatrial septum

De Voogt et al. compared the pacing and sensing properties of leads positioned in the right atrium appendage and the low interatrial septum. The pacing threshold was comparable in both groups. Impedance was higher in the low atrial septum group but the difference was no longer significant after 6 months. Far-field R-wave voltage > 1 mV during ventricular pacing appeared in 87% of the cases in the right atrium appendage group and in 94% of the cases in the low atrial septum group, with the difference showing no clinical significance. The time between the spike of a ventricular pacing stimulus and the sensing of the far-field R-wave in the atrium was longer in the RAA group.[24] The low atrial septum pacing compared to the right atrial appendage pacing had a positive impact on atrial fibrillation prevention. In the study by Minamiguchi et al., after a 1-year follow-up in a group without a history of AF before implantation, 19% of patients paced in the right atrial appendage developed this arrhythmia while in the group paced in the low atrial septum, only 5.9% did so. Also, in the group with a positive history of AF, 22% of the RAA group developed persistent AF, whereas none of the LAS group did.[25]

Hakacova et al. also assessed the properties of septal pacing. They divided patients with conventional indications for pacemaker implantation and recurrent AF into 2 groups: The 1st group had the atrial lead implanted in the atrial septum near the foramen ovale, and the 2nd group received conventional right atrium appendage pacing. The researchers observed no difference in mode-switch episodes and AF burden between the groups, although there was a trend for more AF in the RAA group. A limitation of this study was the small group (44 patients, of whom only 22 completed a 12-month follow-up). Pacing and sensing parameters were comparable in both groups and there were no displacements of electrodes or lead-related complications.[26] Wang et al. compared low atrial septum pacing to right atrial appendage pacing in patients with sinus node disease and paroxysmal AF and found that septal pacing improved atrial mechanical function and synchronized interatrial contraction.[27]

Bachmann's bundle

The Bachmann's bundle is a myocardial tract that originates in the crista terminalis, medial to the superior vena cava, in the proximity of the sinoatrial node, which extends over the atrial roof to the left atrium. It is approximately 3 cm long and in the left atrium it divides into 2 bundles of fibers which distribute to the left atrium. The anterior one advances towards the left atrium appendage, and the posterior one spreads between pulmonary veins. The histological characteristics of the specialized fibers in the Bachmann's bundle resemble those of the Purkinje tissue and they conduct impulses at a higher velocity than the surrounding myocardium, thus being a preferential path for the activation of the left atrium.[28] The Bachmann's bundle is the fastest way to conduct an impulse between both atria. Disturbances in the conduction result in an impaired electrical and mechanical function of the left atrium which may contribute to the occurrence of atrial fibrillation.

In a study by Bailin et al., a group of 63 patients with the atrial lead implanted in the Bachmann's bundle region was compared to 57 patients with the atrial lead in the right atrium appendage. They observed no differences in pacing and sensing properties during the whole follow-up period, however the P-wave duration in the Bachmann's bundle group was shorter compared to the sinus rhythm whilst in the right atrium appendage group it was longer. After 1 year, patients with the right atrium appendage location had a higher rate of a chronic AF (53% compared to

25%). Implantation time was comparable in both groups, which indicates the feasibility and a probable efficacy in attenuating the progression of AF.[29] Bailin et al. also compared the influence of the Bachmann's bundle pacing and the right atrium appendage pacing on the progression to chronic AF in patients with paroxysmal atrial fibrillation without antiarrhythmic pharmacotherapy. The study showed that a significantly greater number of patients in the BB group maintained the sinus rhythm compared to the right atrium appendage group (75 vs 47%). The Bachmann's bundle pacing also resulted in a decrease of P-wave duration compared to both RAA pacing and sinus rhythm, while being safe and feasible.[30]

A positive impact of pacing at the Bachmann's bundle region was also proved in studies by our group. This lead location was shown to improve interatrial conduction, and left ventricle filling, as well as to reduce the need for ventricular pacing with all its adverse effects.[31,32]

Multisite pacing

The concept of multisite pacing, similarly to the Bachmann's bundle pacing, aims to decrease the total atrial activation times.

In the PASTA study, the influence of alternative atrial pacing sites on AF incidence was analyzed. 142 patients were randomized into 4 atrial lead locations: free right atrial wall, right atrium appendage, coronary sinus ostium or dual-site right atrial pacing.[33] AF episodes were taken into account if the time in AF was over 1%. No statistically significant differences were found in AF occurrence and the percentage of atrial or ventricular pacing after 24 months. The AV delay time was programmed to be 30 ms longer than a spontaneous conduction but the percentage of the ventricular pacing was surprisingly high, with values over 60%, but there were no significant differences between groups. Implantation in the coronary sinus ostium and the dual-site implantation showed significantly longer procedure times and a higher rate of dislodgments of atrial electrodes. Ninety percent of patients in this study were free from AF at the enrollment and only 3–6% developed this arrhythmia. This was probably a group with an initially low risk of AF and this study shows that in this group of patients, higher rates of dislodgments and longer radioscopy exposure times are not countervailed by antiarrhythmic advantages.

In the study by Saksena et al., 120 patients with at least 2 symptomatic AF episodes in the preceding 3 months and bradyarrhythmia requiring permanent pacing underwent implantation of a dual-chamber pacemaker with 2 atrial leads in the high atrial septum and in the coronary sinus ostium and then were assigned to different pacing modes: high RA overdrive, dual-site right atrial overdrive or support pacing (DDI or VDI) for 6 months each in a randomized sequence.[34] AF free survival tended to be longer in the dual-site group, and the risk of a recurrence of arrhythmia was lower in the subgroup of antiarrhythmic drug treated-patients with the dual-site overdrive pacing mode, especially in patients with less than 1 AF event per week. However, a comparative analysis of AF prevention between the pacing modes is greatly impacted by poor adherence and high crossover rates in support and overdrive high RA pacing.

Prakash et al. randomized 79 patients in the same way as Saksena et al. and performed echocardiographic studies which proved that the dual-site atrial pacing improved the atrial filling fraction, whilst the high right atrium pacing resulted in a deterioration of the left ventricle fraction.[35]

Delfaut et al., in their study, proved that in patients with drug-refractory recurrent atrial fibrillation and bradycardia, the atrial pacing in combination with antiarrhythmic drugs eliminates or markedly reduces recurrences of arrhythmia.[36] The benefit was enhanced by dual-site right atrial pacing as compared to single-site atrial pacing at the high right atrium and the coronary sinus ostium.

Tse et al., in their study, suggested that overdrive pacing at the right atrial appendage and the coronary sinus ostium simultaneously or pacing the interatrial septum leads to a reduction of the atrial activation time and improves mechanical efficiency, which may slow down the progression of a proarrhythmic substrate in the atria.[37]

Biatrial pacing achieved by simultaneous pacing at the coronary sinus and the high right atrium was investigated by D'Allonnes et al. in a group of 86 patients with tachyarrhythmias and a P-wave duration ≥ 120 ms, or an interatrial conduction time ≥ 100 ms.[38] After 33 months, 64% of patients maintained the sinus rhythm including 32.6% without any documented recurrence of arrhythmia. The same location of atrial leads was used in a study by Lau.[39] In patients with paroxysmal AF without bradycardia, biatrial pacing above the sinus rate prolonged the time to clinical recurrence of arrhythmia compared to high right atrial pacing at 30 beats per minute.

Yu et al. analyzed the atrial conduction time and electrogram width of the right posterior interatrial septum during drive-train stimulation at the high right atrium, the distal coronary sinus or both sites simultaneously and an early extrastimulation.[40] Biatrial pacing reduced the atrial conduction delay caused by the early extrastimulation and reduced the increase of electrogram width at the posterior interatrial septum. These effects could possibly prevent induction of atrial fibrillation. Hansen et al. investigated AF susceptibility in mongrel dogs and concluded that multisite pacing simultaneously at the pulmonary vein ostia, left atrium and right atrium decreased atrial activation times and reduced AF induction by 40%.[41]

Biatrial pacing was compared to right atrial appendage pacing and backup pacing by Levy et al.[42] In the study, the pacing at a base rate of 70 beats/min showed an antifibrillatory effect compared to an inhibited pacing at 40 beats/min, but there was no additional benefit from biatrial pacing compared to single site right atrial pacing.

Lewicka-Nowak et al. described the efficacy and feasibility of atrial resynchronization using multisite atrial pacing at the Bachmann's bundle region and the coronary sinus ostium.[43] In 97 patients with symptomatic, drug-refractory paroxysmal or persistent atrial fibrillation and a sinus P-wave duration ≥ 120 ms, this treatment resulted in a decrease of the incidence and duration of AF-related hospitalizations, lowering the mean number of antiarrhythmic drugs used and the need for cardioversion. In another study of theirs, in patients with similar characteristics, pacing of the Bachmann's bundle and the coronary sinus ostium had no influence on the global hemodynamics, nevertheless it caused an earlier and more synchronized LA contraction and a reversed right-left contraction sequence.[44]

Despite the encouraging results of some research projects, the usefulness of multisite pacing may be limited by the requirement for 2 atrial leads and a Y connector.

Conclusions

Atrial pacing is a method of choice in sinoatrial node disease. It provides the physiological electrical activation of the atria and, in the presence of proper atrioventricular conduction, it enables the physiological activation of the ventricles. Historically, the atrial pacing lead was located within the appendix of the right atrium, which was constrained by the lead fixation technique. The introduction of active fixation leads enabled almost all the atrial locations to be paced and the electrical and mechanical properties of such pacing sites are of paramount importance. The overview of the published studies makes it possible to draw a conclusion that the upper and more central locations within the right atrium seem to be superior to the lower and more lateral pacing sites.

The pacing sites incorporating the right atrial roof, the upper part of the interatrial septum and the Bachmann's bundle itself make possible fast interatrial conduction and shorter P-wave duration, resulting in better antiarrhythmic properties. More physiological impulse propagation seems to prevent the long-term remodeling of the atria. Multisite atrial pacing, despite its positive influence on atrial synchrony, does not seem to be widely used due to the adverse side effects caused by numerous intravenous electrodes.

The vast area of uncertainty regarding the long term hemodynamic effects of different atrial pacing sites originates from the lack of large studies with longer follow-up. Atrial arrhythmias may not be a reliable end-point in this regard because other factors influence the arrhythmogenesis to a large extent. There is a need for prospective follow up studies assessing the contribution of different atrial pacing sites to the development of so-called heart failure with preserved systolic function or even simple long-term observations on left atrium volume.

References

1. Roithinger FX, Cheng J, Sippens-Groenewegen A, et al. Use of electroanatomic mapping to delineate transseptal atrial conduction in humans. *Circulation*. 1999;100:1791–1797.
2. Lemery R, Soucie L, Martin B, Tang AS, Green M, Healey J. Human study of biatrial electrical coupling: Determinants of endocardial septal activation and conduction over interatrial connections. *Circulation*. 2004;110:2083–2089.
3. Betts TR, Roberts PR, Morgan JM. High-density mapping of left atrial endocardial activation during sinus rhythm and coronary sinus pacing in patients with paroxysmal atrial fibrillation. *J Cardiovasc Electrophysiol*. 2004;15:1111–1117.
4. O'Neal WT, Almahmoud MF, Soliman EZ. Resting heart rate and incident atrial fibrillation in the elderly. *Pacing Clin Electrophysiol*. 2015;38:591–597.
5. Uhm JS, Mun HS, Wi J, et al. Prolonged atrial effective refractory periods in atrial fibrillation patients associated with structural heart disease or sinus node dysfunction compared with lone atrial fibrillation. *Pacing Clin Electrophysiol*. 2013;36:163–171.
6. Hoffmann E, Sulke N, Edvardsson N, et al. Atrial fibrillation therapy trial investigators. New insights into the initiation of atrial fibrillation: A detailed intraindividual and interindividual analysis of the spontaneous onset of atrial fibrillation using new diagnostic pacemaker features. *Circulation*. 2006;113:1933–1941.
7. Andersen HR, Nielsen JC, Thomsen PE, et al. Long-term follow-up of patients from a randomised trial of atrial versus ventricular pacing for sick-sinus syndrome. *Lancet*. 1997;350:1210–1216.
8. Kerr CR, Connolly SJ, Abdollah H, et al. Canadian trial of physiological pacing: Effects of physiological pacing during long-term follow-up. *Circulation*. 2004;109:357–362.
9. Hjortshøj S, Riahi S, Nielsen JC, Skjøth F, Lundbye-Christensen S, Andersen HR. DANPACE Investigators. Does atrial pacing lead to atrial fibrillation in patients with sick sinus syndrome? Insights from the DANPACE trial. *Europace*. 2014;16:241–245.
10. Elkayam LU, Koehler JL, Sheldon TJ, Glotzer TV, Rosenthal LS, Lamas GA. The influence of atrial and ventricular pacing on the incidence of atrial fibrillation: A meta-analysis. *Pacing Clin Electrophysiol*. 2011;34:1593–1599.
11. Inoue N, Ishikawa T, Sumita S, et al. Suppression of atrial fibrillation by atrial pacing. *Circ J*. 2006;70:1398–1401.
12. Nielsen JC, Thomsen PE, Højberg S, et al. DANPACE Investigators. A comparison of single-lead atrial pacing with dual-chamber pacing in sick sinus syndrome. *Eur Heart J*. 2011; 32:686–696.
13. Kuniewicz M, Rydlewska A, Karkowski G, Lelakowska-Pieła M, Majewski J, Lelakowski J. Effectiveness of atrial versus atrioventricular pacing for sick sinus syndrome during long-term follow-up. *Kardiol Pol*. 2015;73:7–16.
14. Nielsen JC, Thomsen PE, Højberg S, et al. DANPACE investigators. Atrial fibrillation in patients with sick sinus syndrome: The association with PQ-interval and percentage of ventricular pacing. *Europace*. 2012;14:682–689.
15. Veasey RA, Arya A, Freemantle N, et al. The usefulness of minimal ventricular pacing and preventive AF algorithms in the treatment of PAF: The "MinVPace" study. *J Interv Card Electrophysiol*. 2010;28: 51–57.
16. Bennett D. Comparison of the acute effects of pacing the atrial septum, right atrial appendage, coronary sinus os, and the latter two sites simultaneously on the duration of atrial activation. *Heart*. 2000;84:193–196.
17. Katsivas A, Manolis AG, Lazaris E, Vassilopoulos C, Louvros N. Atrial septal pacing to synchronize atrial depolarization in patients with delayed interatrial conduction. *Pacing Clin Electrophysiol*. 1998;21: 2220–2225.
18. Healey JS, Israel CW, Connolly SJ, et al. Relevance of electrical remodeling in human atrial fibrillation: Results of the asymptomatic atrial fibrillation and stroke evaluation in pacemaker patients and the atrial fibrillation reduction atrial pacing trial mechanisms of atrial fibrillation study. *Circ Arrhythm Electrophysiol*. 2012;5:626–631.
19. Kristensen L, Nielsen JC, Mortensen PT, et al. Sinus and paced P wave duration and dispersion as predictors of atrial fibrillation after pacemaker implantation inpatients with isolated sick sinus syndrome. *Pace*. 2004;27:606–614.

20. De Sisti A, Leclercq JF, Stiubei M, Fiorello P, Halimi F, Attuel P. P Wave duration and morphology predict atrial fibrillation recurrence in patients with sinus node dysfunction and atrial-based pacemaker. *PACE*. 2002;25,1546–1554.
21. Bayés de Luna A, Cladellas M, Oter R, et al. Interatrial conduction block and retrograde activation of the left atrium and paroxysmal supraventricular tachyarrhythmia. *Eur Heart J*. 1988;9:1112–1118.
22. Endoh Y, Nakamura A, Suzuki T, et al. Clinical significance of prolonged P wave width after right atrial appendage pacing In sick sinus syndrome. *Circ J*. 2003;67:485–489.
23. Stabile G, Senatore G, De Simone A, et al. Determinants of efficacy of atrial pacing in preventing atrial fibrillation recurrences. *J Cardiovasc Electrophysiol*. 1999;10:2–9.
24. de Voogt WG, van Mechelen R, van den Bos A, Scheffer M, van Hemmel NM, Levine PA. Electrical characteristics of low atrial septum pacing compared with right atrial appendage pacing. *Europace*. 2005;7:60–66.
25. Minamiguchi H, Nanto S, Onishi T, Watanabe T, Uematsu M, Komuro I. Low atrial septal pacing with dual-chamber pacemakers reduces atrial fibrillation in sick sinus syndrome. *J Cardiol*. 2011;57:223–230.
26. Hakacova N, Velimirovic D, Margitfalvi P, Hatala R, Buckingham TA. Septal atrial pacing for the prevention of atrial fibrillation. *Europace*. 2007;9;1124–1128.
27. Wang M, Siu CW, Lee KL, et al. Effects of right low atrial septal vs right atrial appendage pacing on atrial mechanical function and dyssynchrony in patients with sinus node dysfunction and paroxysmal atrial fibrillation. *Europace*. 2011;13(9:1268–1274.
28. Savitt MA, Rankin JS. Use of Bachmann's bundle for bipolar atrial pacing. *Ann Thorac Surg*. 1993;56:183–184.
29. Bailin SJ, Adler S, Giudici M. Prevention of chronic atrial fibrillation by pacing in the region of Bachmann's bundle: Results of a multicenter randomized trial. *J Cardiovasc Electrophysiol*. 2001;12:912–917.
30. Bailin S. Is Bachmann's bundle the only site for single-site pacing to prevent atrial fibrillation? Results of multicenter randomized trial. *Card Electrophysiol Rev*. 2003;7:325–328.
31. Sławuta A, Skoczyński P, Kliś M, et al. Bachmann's bundle pacing improves left ventricle filling and reduces the need for ventricular pacing. *Eur Heart J*. 2014;35:90(Abstract suppl).
32. Sławuta A, Skoczyński P, Bańkowski T, Kliś M, Gajek J. Bachmann's bundle pacing not only improves interatrial conduction but also reduces the need for ventricular pacing. *Eur Heart J*. 2013;34:489(Abstract suppl).
33. Spitzer S, Wacker P, Gazarek S, Malinowski K, Schibgilla V. Primary prevention of atrial fibrillation: Does the atrial lead position influence the incidence of atrial arrhythmias in patients with sinus node dysfunction? Results from PASTA study. *PACE*. 2009;32:1553–1561.
34. Saksena S, Prakash A, Ziegler P, et al. Improved suppression of recurrent atrial fibrillation with dual-site right atrial pacing and antiarrhythmic drug therapy. *J Am Coll Cardiol*. 2002;40:1140–1150.
35. Prakash A, Saksena S, Ziegler PD, et al. Dual site right atrial pacing can improve the impact of standard dual chamber pacing on atrial and ventricular mechanical function in patients with symptomatic atrial fibrillation: Further observations from the dual site atrial pacing for prevention of atrial fibrillation trial. *J Interv Card Electrophysiol*. 2005;12:177–187.
36. Delfaut P, Saksena S, Prakash A, Krol RB. Long-term outcome of patients with drug refractory atrial flutter and fibrillation after single- and dual-site right atrial pacing for arrhythmia prevention. *J Am Coll Cardiol*. 1998;32:1900–1908.
37. Tse HF, Hettrick DA, Mehra R, Lau CP. Improved atrial mechanical efficiency during alternate- and multiple-site atrial pacing compared with conventional right atrial appendage pacing: implications for selective site pacing to prevent atrial fibrillation. *J Am Coll Cardiol*. 2006;47:209–212.
38. D'Allonnes GR, Pavin D, Leclercq C, et al. Long-term effects of biatrial synchronous pacing to prevent drug-refractory atrial tachyarrhythmia: A nine-year experience. *J Cardiovasc Electrophysiol*. 2000;11:1081–1091.
39. Lau CP, Tse HF, Yu CM, et al. New indication for preventive pacing in atrial fibrillation (NIPP-AF) investigators. Dual-site atrial pacing for atrial fibrillation in patients without bradycardia. *J Am Cardiol*. 2001;88:371–375.
40. Yu WC, Chen SA, Tai CT, Feng AN, Chang MS. Effects of different atrial pacing modes on atrial electrophysiology: Implicating the mechanism of biatrial pacing in prevention of atrial fibrillation. *Circulation*. 1997;96:2992–2996.
41. Hansen JC, Latchamsetty R, Lavi N, et al. High-density biatrial pacing protects against atrial fibrillation by synchronizing left atrial tissue. *J Interv Card Electrophysiol*. 2010;27:81–87.
42. Levy T, Walker S, Rochelle J, Paul V. Evaluation of biatrial pacing, right atrial pacing, and no pacing in patients with drug refractory atrial fibrillation. *Am J Cardiol*. 1999;84:426–429.
43. Lewicka-Nowak E, Kutarski A, Dabrowska-Kugacka A, Rucinski P, Zagozdzon P, Raczak G. A novel method of multisite atrial pacing, incorporating Bachmann's bundle area and coronary sinus ostium, for electrical atrial resynchronization in patients with recurrent atrial fibrillation. *Europace*. 2007;9:805–811.
44. Lewicka-Nowak E, Dabrowska-Kugacka A, Rucinski P, Kozlowski D, Raczak G, Kutarski A. Atrial function during different multisite atrial pacing modalities in patients with bradycardia–tachycardia syndrome. *Circ J*. 2009;73:2029–2035.

The pathogenesis and treatment of polycystic ovary syndrome: What's new?

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Abstract

Polycystic ovary syndrome (PCOS) is currently the leading cause of menstrual complications in women. It is characterized by clinical and/or biochemical hyperandrogenism, ovulation abnormalities and the presence of enlarged and/or polycystic ovaries in ultrasound images (12 or more small bubbles located circumferentially and/or ovarian volume > 10 mL). It is often comorbid with hyperinsulinemia, dyslipidemia, overweight or obesity, and is a risk factor for the development of diabetes and cardiovascular diseases (CVDs). The treatment of patients with PCOS depends on the prevailing symptoms. The aim of this paper is to present the etiopathogenesis, clinical and biochemical implications, and non-pharmacological and pharmacological treatment options – those approved by worldwide scientific organizations as well as new therapies whose initial results are encouraging enough to prompt researchers to explore them further.

Key words: treatment, statins, metformin, PCOS

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Polycystic ovary syndrome (PCOS), also known as the Stein-Leventhal syndrome, is one of the most common endocrinopathies among women of reproductive age. It is estimated that it affects 3–15% of all women. An abnormality in the ovaries is the primary cause of the disorder, but additional agents, such as obesity and environmental factors, affect the development of individual symptoms.[1] The Rotterdam criteria (2003) are the most widely used and relevant criteria for the diagnosis of PCOS. The disorder is diagnosed if 2 of the 3 specified conditions are met: (1) hyperandrogenism (detected by clinical and/or biochemical testing) (2) ovulation abnormalities, and/or (3) 12 or more cysts on one ovary and/or ovarian volume > 10 mL. There are also 2 other definitions of the syndrome in addition to the Rotterdam criteria. According to the criteria proposed by the National Institutes of Health (NIH, 2009), a diagnosis of PCOS involves detection of clinical or biochemical hyperandrogenism and chronic ovulation disorders. The Androgen Excess Society (2006), on the other hand, treats hyperandrogenism as the basic PCOS disorder and the prerequisite for a diagnosis, in combination with one of the 2 remaining Rotterdam criteria.[2] In all these cases, PCOS can be diagnosed after Cushing's syndrome, congenital adrenal hyperplasia and/or androgen-secreting tumors have been ruled out.

Based on the Rotterdam criteria, 4 phenotypes of PCOS can be distinguished: (1) classic: hyperandrogenism (H), ovulation disorders (O) and a polycystic ovary (P) detected by USG (HOP); (2) with hyperandrogenism and ovulation disorders, but with a normal ovarian USG image (HO); (3) with hyperandrogenism and a polycystic ovary USG image, but without ovulation disorders (HP); (4) with ovulation disorders and a polycystic ovary USG image, but without evidence of hyperandrogenism (OP).[1–3]

Despite these seemingly clear criteria, the etiology of PCOS remains unknown, and precise treatment procedures have not been established. PCOS therefore continues to be the object of research and scientific inquiry. In this paper, the postulated causes and possible effects of the clinical and biochemical syndrome are discussed, along with currently accepted and novel therapeutic methods.

Etiopathogenesis

A defect of the ovarian cells (most likely theca cells) is the underlying cause of PCOS, resulting in excessive androgen synthesis and the clinical and biochemical symptoms of the disease.[1,2] In the literature, reference is made to the participation of genetic factors, including ethnicity; there is a higher frequency of PCOS in Spanish, native American and Mexican women.[41] In their original description of the syndrome, Stein and Leventhal emphasized that a high ratio of luteinizing hormone (LH) to follicle-stimulating hormone (FSH) is one of the basic disorders. It has also been suggested that the underlying causes of PCOS include increased frequency of gonadotropin-releasing hormone (GnRH) pulses that stimulate the theca cells to produce androgen; decreased levels of FSH (and thus a defect in the late luteal and early follicular phases); insulin resistance via a post-receptor defect in the fat tissue and skeletal muscles (abnormal phosphorylation of tyrosine kinase); pancreatic beta-cell dysfunction; and obesity.[1,3] It is often impossible to determine definitively what is a cause and what is an effect in the development of PCOS. In addition, it is generally recognized that obesity increases menstrual disorders and hyperandrogenism, while weight reduction reduces the clinical signs. Reduced insulin sensitivity is an important issue in both obese and underweight women with PCOS; it is estimated that 50–70% of women with the condition show insulin resistance of varying intensities.[3]

Genetic factors

The influence of genetic factors was highlighted by Davies et al., who proved that mothers of women with PCOS are more likely to have a cardiovascular disease and that their risk of hypertension is twice as high as mothers of women without PCOS, while fathers of women with PCOS are twice as likely to have heart disease and 4 times more likely to have experienced cerebral stroke.[5] Tan et al. emphasized the increased likelihood of insulin resistance (IR) associated with certain genes (such as *INSIG2* and *MC4R)* and the particular impact of TCF7L2 SNP on the development of diabetes mellitus type 2 (DM2) and body weight gain in patients with PCOS (a per-allele weight gain of 1.56 kg/m^2).[6] The etiology of IR was also discussed by Fica et al., who, while highlighting the complex mechanisms of PCOS, identified insulin receptor autophosphorylation, reduced levels of phosphatidylinositol-3-kinase in muscle tissue and visceral adiposity as probable mechanisms.[7]

Hyperinsulinemia/insulin resistance

Hyperinsulinemia in combination with pancreatic beta cell dysfunction results in an increased risk of many diseases, including type 2 diabetes, hypertension, dyslipidemia, endothelial dysfunction, atherosclerosis and cardiovascular diseases. Insulin also stimulates the theca cells of the ovary to produce excessive testosterone, which is responsible for the clinical symptoms of hyperandrogenism (acne, hirsutism, alopecia).[8] Cardiovascular risk is also elevated in women who are chronic smokers, as demonstrated in a recent work by Marotti et al.[4]

Inflammation

The role of inflammation in PCOS has been the subject of a number of studies, and direct correlations have been found between increased levels of inflammation markers (CRP, ferritin, leukocyte TNF-α, IL-6, IL-18) and the development of PCOS. Other contributors include elevated levels of PAI1 and free fatty acids, influencing excessive phosphorylation of serine residues, leading to a rise in insulin resistance.[9] Newly emerging issues include a pathogenic correlation of the markers of iron overload with PCOS. Increased levels of ferritin and transferrin and a higher frequency of the HP2/HP2 genotype of the haptoglobin α chain have been observed, causing a reduction of anti-inflammatory cytokines and antioxidant molecules, leading to a state of chronic inflammatory response.[10,11]

Clinical implications

In addition to hyperandrogenism and its related complications, the most common abnormalities associated with PCOS include menstrual disorders (amenorrhea or oligomenorrhea), often leading to infertility (in 73–74% of the cases), abdominal obesity (30–70%) and type 2 diabetes (approximately 10%).[1,7] The prevalence of metabolic disorders in women with PCOS is higher than in the healthy population, with type 2 diabetes occurring at the highest frequency (3–7 times), especially in Indians.[2,44] Other frequent disorders include hirsutism (85–90%) symptoms of metabolic syndrome (MS, approximately 40%), obesity/overweight (40–60%), lipid disorders, arterial hypertension (approximately 20%) (Table 1). It is worth noting that any excessive weight in these patients impairs the regularity of menstrual bleeding and responses to metformin and insulin treatment, exacerbates the symptoms of hyperandrogenism and also increases cardiovascular risk (Fig. 1).[3,11,15]

Recent reports have revealed a close correlation between mental disorders and clinical PCOS symptoms such as acne, hirsutism, infertility, obesity and a poorer quality of life. A higher frequency of depression, drug-related and bipolar disorders, bulimia, anorexia or non-spe-

Table 1. Frequency of typical disorders in PCOS

Abnormality	Frequency
Infertility	73–75%
Hirsutism	85–90%
Metabolic syndrome: abdominal obesity diabetes type 2 arterial hypertension disturbed lipid metabolism	40% 30–70% 10% 20% unavailable

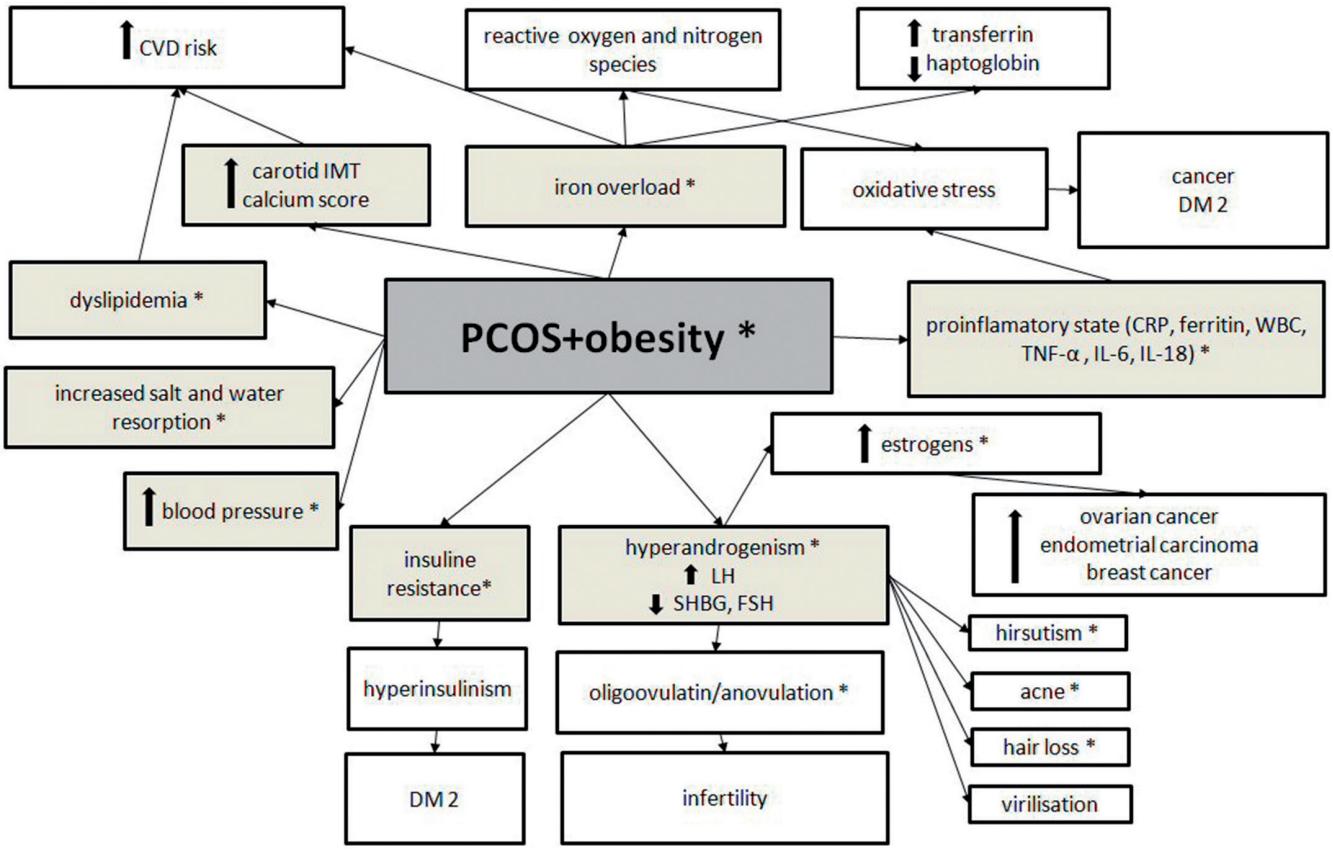

Fig. 1. A chart of correlated symptoms in obese PCOS patients and possible targets for treatment (*)

cific dietary disorders was noted among PCOS patients.[12] Chronic hyperandrogenemia, which leads to increased aromatization of androgens to estrogens in adipose tissue, may contribute further to the development of hormone-dependent tumors, such as endometrial, mammary or ovary neoplasms.[13]

Taking into account the considerations presented above, it is advisable for routine oral glucose tolerance testing (OGTT) to be performed in obese women with PCOS (but routine OGTT is not necessary for women with normal body weight). Patients diagnosed with PCOS are included in the groups at risk of developing diabetes, in accordance with the standards of the Polish Diabetes Association.[14]

Biochemical disorders

In polycystic ovaries, abnormal steroidogenesis is manifested primarily by increased production of androgens and estradiol, and the malfunctioning hypothalamic-pituitary-ovarian axis is manifested by increased secretion of LH, anti-mullerian hormone (AMH), a higher frequency of GnRH pulses and a reduction in FSH concentration.[1,2] These correlations (most importantly the participation of androgens) are associated with a disturbed lipid profile: an increase in very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and triglycerides (TG), and a decrease in HDL- and LDL-cholesterol, regardless of body weight. Dyslipidemia, coagulation disorders, increased plasminogen activator inhibitor 1 (PAI1) and other metabolic consequences, increases in the coronary artery calcium score and resultant increases in carotid intima-media thickness (CIMT) lead to an increase in the risk of cardiovascular disorders.[15] Obesity/overweight coexisting with PCOS can lead to iron deficiency (through increased production of proinflammatory cytokines, oxidative stress and a resultant increase in the levels of hepcidin, inhibiting the absorption of iron from enterocytes), and thus the signs of anemia in those women. There are many reports on the role of iron deficiency in the development of diabetes and its complications. The opposite situation also occurs, and iron overload in obese women with PCOS as measured by levels of ferritin, soluble transferrin receptor (sTfR), hepcidin and heme iron, is also a risk factor for insulin resistance, type 2 diabetes and cardiac disease. Reducing the consumption of red meat and the use of iron-zinc chelators may be beneficial.[10] Bu et al. have shown a relationship between elevated serum levels of preptin (34-amino acid protein secreted from the beta cells of the pancreas along with insulin) with impaired glucose tolerance (IGT) in both PCOS patients and healthy controls, but found no correlation with PCOS status.[16]

The results of the available studies suggest that PCOS entails subclinical damage at the cellular level at an early stage of the disease, with the possibility of many adverse long-term sequelae. This indicates that it is necessary to implement prevention strategies and interventions that must sometimes be started at an early stage in the development of the symptoms.

Currently applied treatment

The procedures used in PCOS treatment depend primarily on the desired clinical effect: infertility treatment, regulation of menstrual disturbances, alleviation of the symptoms of hyperandrogenism or obesity treatment.

For women wishing to conceive, clomifene still remains first-line therapy. It is an estrogen receptor modulator that directly affects the hypothalamic-pituitary axis, acting rapidly and effectively; 75% of the pregnancies in patients using clomifene are conceived in the first 3 months of treatment.[1]

Another drug used with PCOS patients wishing to restore fertility is metformin; its effectiveness can be observed after 6 months of treatment.[1,2] The role of metformin in inducing ovulation is still controversial. It is known for certain that metformin contributes to reducing insulin levels and androgens, thus restoring the regularity of the ovulatory cycle and periods. Metformin is a biguanide derivative, well-known for many years; it not only reduces insulin resistance and blood pressure, improves the lipid profile and antioxidant characteristics and increases the levels of sex hormone binding globulin (SHBG), but also (through its pleiotropic effect on the vascular endothelium) acts to protect the cardiovascular system.[21,22] In addition, its dose-dependent protective effect against the risk of developing endometrial, mammary, intestinal and hepatic cancer has been reported by several authors.[19,20] Although many researchers claim that metformin plays a role in reducing body weight, a recent study has contradicted that view, pointing to the absence of any such correlation; the only possible relationship involves redistribution of active visceral fat to inactive subcutaneous fat.[21] The starting dose of 500 mg once a day at lunch time, increased to 3 tablets/24 h when tolerance is good, does not influence insulin resistance if it is not applied too long.[1] Tang et al. have shown a negative correlation in the reduction of insulin resistance after 6 months of therapy, as opposed to a study by Oppelt et al. that found increased insulin resistance after a 2-year period of intervention.[22,23] Although older reports did not confirm the effect of metformin on clinical symptoms related to hyperandrogenism, recent data indicate effects of that kind, especially in reducing skin problems (hirsutism, acne, acanthosis nigricans).[1,12]

Other therapeutic options for women with the problem of infertility in PCOS are gonadotropins, used to induce ovulation, or laparoscopic surgery for patients resistant to pharmacological therapies. Laparoscopic techniques that

can successfully trigger ovulation include ovarian biopsy and electrocautery, laparoscopic ovarian drilling, transvaginal hydrolaparoscopy, ultrasound guided transvaginal ovarian needle drilling or laparoscopic ovarian multi-needle intervention.[1,43]

PCOS patients whose goal is not pregnancy are usually advised to use oral contraceptives (OCP), which effectively restore the rhythm of bleeding, reduce hyperandrogenism and also contribute to reducing the risk of endometrial hyperplasia.[1] The effects of excessive androgen synthesis on a patient's appearance – hirsutism, acne, alopecia, acanthosis nigricans – are among the most frequent problems causing patients with PCOS to seek medical care. They are also among the main causes of deterioration in their quality of life, chronic stress and mental disorders, including depression.[12] The pharmacological therapies for these problems primarily make use of antiandrogen drugs, such as cyproterone acetate in combination with ethinyl estradiol (OCP), spironolactone or metformin.

Drugs that are not normally used in the treatment of hyperandrogenism, but which can be taken into consideration, include long-acting GnRH analogs, ketoconazole, glucocorticoids, flutamide and finasteride.

Local procedures intended to reduce excessive hair growth include beauty treatments, such as hair removal, electrolysis, laser destruction of the follicles and/or application of a cream containing 13.9% ornithine decarboxylase inhibitor and 13.9% eflornithine. Studies have shown that the substances contained in the cream, applied twice a day for 6 months reduce hair growth. The cream is well tolerated and, in combination with laser epilation, is more effective than hair removal methods used alone.[25]

Another goal of therapy in PCOS is to reduce obesity, where in addition to lifestyle modification, diet and pharmacological therapy with metformin, surgical methods have been applied. It has been demonstrated that bariatric procedures for the treatment of obesity greatly improve the profile of patients with PCOS by preventing metabolic syndrome; reducing body weight, blood pressure and the risk of cardiovascular diseases; restoring normal function of the hypothalamic-pituitary axis; improving reproductive function; and also, as demonstrated by Eid et al., normalizing blood pressure in approximately 78% of treated patients with previous hypertension.[24]

New therapeutic options

Among the new therapeutic options for PCOS patients, isotretinoin – a popular drug used to treat acne – deserves to be mentioned. In a study by Cakir et al., women with acne (46 without PCOS and 50 with PCOS) received intramuscular injections of 0.5–1 mg/kg/dL isotretinoin, and the effects of the treatment were observed after one and 2 years. In both groups, the therapy was highly effective (91.6% achieved complete remission of their acne), which may lead to the use of isotretinoin as a first line treatment for PCOS patients with acne, second only to oral contraceptive therapy. Isotretinoin may improve the patients' reduced AMH levels, which correlate with elevated androgen levels.[26] However, the results of a recently published study by Cetinözman et al. indicate that severe acne does not correlate with the level of androgens or sensitivity to insulin. Isotretinoin therapy not only fails to produce the desired clinical effect but contributes to an increase in body weight and triglyceride levels in the patients.[27] Due to the high costs, the multitude of potential adverse effects and its problematic effectiveness, isotretinoin treatment is not yet widely recommended in PCOS, although the prospects are promising.

Androgenetic alopecia (AGA) is another problem associated with PCOS. Antiandrogenic drugs (cyproterone acetate) and inhibitors of 5-alpha reductase (finasteride) are particularly effective in hyperandrogenism accompanied by increased body mass, but a good response has also been observed with a 2% solution of a drug called minoxidil used twice a day for at least 6 months. This chemical, after it is converted to its active form (minoxidil sulfate), opens the ATP-dependent potassium channels in cells, causing a vasodilatation effect by increasing the production of vascular endothelial growth factor (VEGF) in dermal papillae, stimulating hepatocyte growth factor (HGF) production and activating the synthesis of prostaglandins, which are mechanisms that ultimately lead to stimulation of hair growth.[28]

Other therapeutic options include combination therapy. An interesting alternative was proposed recently by Vinaixa et al., who subjected non-obese women with PCOS to 3-month flutamide-metformin-pioglitazone polytherapy combined with ester-progestogen treatment, pointing to the benefit of such treatment with respect to the lipid profile (reduced LDL, increased HDL) higher androgen levels as well as increased carotid intima media (CIM) thickness, which in turn prevents the occurrence of atherosclerosis and related complications.[29]

Another alternative is the use of thiazolidinedione derivatives, which stimulate peroxisome proliferator-activated receptor type γ (PPAR-γ), enhance insulin sensitivity, reduce the level of glucose. However, these drugs do not reduce androgen levels and can contribute to patients' weight gain; in addition, they are contraindicated for women wishing to become pregnant.[30]

Encouraging results suggest that metformin combination therapy with new drugs acting on the incretin system (glucagon-like peptide receptor agonists 1-GLP-1, for example liraglutide or exenatide) leads to more effective weight reduction, lowers insulin resistance and improves reproductive function. However, they are still not registered as drugs with a high safety profile in women of reproductive age.[31]

Several studies have recently been published on the effectiveness of vitamin D supplementation, especially in

autumn and winter. The authors have pointed out that vitamin D deficiency has an impact on the pathogenesis of insulin resistance in PCOS.[32]

Studies on the use of statins for the treatment of PCOS seem to be extremely encouraging, especially considering that approximately 70% of women with this syndrome are affected by lipid disorders and obesity. As blockers of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, statins have pleiotropic effects through their anti-inflammatory, antioxidant, antiproliferative and lipid-lowering activity. By blocking the formation of the malonic acid required for the synthesis of cholesterol, they inhibit the proliferation of cells in the ovarian theca cell layer, with a resultant reduction in the synthesis of steroid hormones.[1] This was confirmed by the results of Celik and Acbay, who demonstrated that a 12-week combination therapy with metformin and rosuvastatin is more effective in reducing the testosterone, DHEA-S, body weight, CRP, TG and LDL cholesterol than the use of either of those drugs alone.[33] Other authors compared the effects of different types of statins on selected endpoints. They proved, for example, that atorvastatin is more effective than simvastatin in improving insulin sensitivity, reducing the level of insulin in the fasting state, lowering blood pressure and reducing the concentration of advanced glycation end products (AGEs, which can affect the course of many diseases and degenerative processes). Atorvastatin also reduces the level of serum malondialdehyde (MDA) as a marker of oxidative stress. MDA is indirectly connected with the reduction of levels of CRP and total testosterone, and with increasing levels of 25-hydroxyvitamin D (25OHD) in PCOS patients.[34,35]

An interesting issue is the finding that supplementation with omega-3, α-lipoic acid and N-acetyl cysteine results in an antioxidant, anti-inflammatory effect, improves insulin sensitivity and the lipid profile of women with PCOS.[36] Salehpour et al. demonstrated that N-acetyl cysteine lowers levels of testosterone, reduces androgen response to gonadotropin stimulation, improves ovulation rates and long-term health after 6 months of treatment.[37]

In recent years, attention has also been paid to the role of increased activity of the sympathetic nervous system in the pathogenesis, progression and treatment of PCOS. It has been shown that increased intraovarian production of nerve growth factor (NGF) and elevated muscle sympathetic nerve activity (MSNA) stimulate the development of obesity, hyperinsulinemia, obstructive sleep apnea (OSA) and metabolic disorders in PCOS patients. In these cases the use of non-pharmacological interventions (weight reduction, continuous positive airway pressure in OSA, electroacupuncture stimulation of baroreceptors), pharmacological treatment (drugs that increase insulin sensitivity) and surgical procedures (renal denervation) can bring surprising results.[38,39]

Studies on the role of fibroblast growth factors (FGFs) in the pathogenesis of PCOS, metabolic disorders, type 2 diabetes and the cutaneous manifestations of hyperandrogenism represent an absolutely new approach. It has been shown that FGFs – particularly FGF-1, -10, -19 and -21 – are involved not only in the regulation of carbohydrate and lipid metabolism and show cardioprotective activity (FGF-21), but are also responsible for the cutaneous manifestations of excessive activity of the sebaceous glands.[40] Decreased levels of FGF-19 have been observed among PCOS patients. An analog of FGF-21 called LY 2405319, currently being tested in clinical settings, reduces insulin resistance and lowers blood glucose, cholesterol, triglycerides, LDL, and also helps in weight reduction.[41]

In recent years, there have been reports concerning improvement in insulin sensitivity in women with PCOS due to myo-inositol treatment. It has been shown in some clinical trials that myo-inositol not only decreases glycemia in OGTT, the homeostatic model assessment (HOMA) index, and reduces the secretion of LH, DHEA, testosterone and progesterone but also improves the serum lipid profile (cholesterol, triglycerides).[42] A high concentration of myo-inositol in the follicular microenvironment improves the availability of oocytes. Therefore myo-inositol restores menstrual regularity, ovulation and effectively increases the chances of getting pregnant. However, further studies are needed to be able to use myo-inositol for fertility treatment.[42]

Table 2 presents a summary of current and new medications, with their contraindications and possible side effects.

Table 2. Drug treatments for PCOS

Drug	Effects	Contraindications	Side effects
Metformin	– restores regular bleeding and ovulation – reduces insulin resistance – improves arterial tension values – improves lipid profile – shows antioxidant activity – increases sex hormone binding globulin (SHBG) level – may help reduce body weight	– hypersensitivity – renal insufficiency – acute or chronic diseases that may cause tissue hypoxia, such as cardiac or respiratory insufficiency – lactation – hepatic damage	– gastrointestinal disorders – lactic acidosis – dyspepsia, diarrhea, nausea, flatulence – metallic aftertaste in the mouth

Table 2. Drug treatments for PCOS – cont.

Drug	Effects	Contraindications	Side effects
Oral Contraceptives	– restore regular periods – reduce symptoms of hyperandrogenism – reduce risk of endometrial hyperplasia	– past or current thromboembolic complications, cerebro- or cardiovascular disorders – obesity (BMI over 30 kg/m^2) – pregnancy or suspected pregnancy – valvular heart disease – active hepatic disease – mammary or uterine cancer – reproductive tract bleeding of unknown etiology – estrogen-dependent tumors	– arterial hypertension – nausea, vomiting – headache – dermal lesions (acne, hirsutism) – body weight gain – turgid breasts – leg cramps – vaginal staining or bleeding
Clomifene	– infertility treatment (ovulation induction)	– allergy to clomifene – pregnancy – hepatic disease – primary hypopituitarism – disturbed thyroid or adrenal function – uterine bleeding of unknown etiology – hormone-dependent tumors	– headache, vertigo – tiredness – disturbed vision – nausea, vomiting – vasomotor symptoms – facial flush – mastalgia – abdominal pain – paramenia
Eflornithine	– controls facial hirsutism	– hypersensitivity to eflornithine or any adjuvant	– acne – chronic folliculitis barbae – alopecia – skin burning sensation – xerodermia – itching – erythema – skin formication
GnRH analogs	– inhibit androgens	– hypersensitivity to any component of the product or other GnRH analogues – pregnancy or lactation – metabolic disorders of the skeletal system	– menopausal symptoms – loss of bone mass – vaginal dryness – insomnia, mood swings, depression – reduced libido
Ketoconazole	– inhibits androgens	– acute or chronic hepatic disease – pregnancy or lactation	– nausea – alopecia – dry skin – uterine bleeding – headache
Steroids	– inhibit androgens	– contraindicated in patients affected by symptoms or diseases which may be a side effect of their use, e.g. diabetes, hypertension, infections	– adrenal suppression – infections – neuro-psychiatric abnormalities – carbohydrate metabolism disorders, diabetes – electrolyte imbalance – osteoporosis – changes in lipid and protein metabolism – Cushing's syndrome – gastric ulcer – muscle weakness – growth disorders in children – glaucoma – menstrual disorders
Spironolactone	– inhibits androgens	– hyperkalemia – touch-sensitive nipples – mastalgia – menstrual disorders – hirsutism – agranulocytosis – headaches – sleepiness – ataxia	– hypersensitivity – hyponatremia, hyperkalemia – primary adrenal insufficiency – severe renal and hepatic failure – acute renal failure
Flutamide	– inhibits androgens	– hypersensitivity to any component of the product	– gynecomastia, mastalgia, galactorrhea – diarrhea, nausea, vomiting, increased appetite – insomnia, fatigue – abnormal liver function

Table 2. Drug treatments for PCOS – cont.

Drug	Effects	Contraindications	Side effects
Finasteride	– reduces alopecia	– hypersensitivity to any component of the product – pregnancy, planned pregnancy or breast-feeding	– decreased libido – rash – enlarged and tender breasts – hypersensitivity reactions
Isotretinoin	– severe acne (due to reduced AMH levels)	– hypersensitivity to isotretinoin, peanuts, soybeans or other ingredients – pregnancy or lactation – liver failure – hypervitaminosis A – increased blood lipid levels – use of tetracycline antibiotics	– dry skin and eyes with conjunctivitis – dry mucous membranes, cheilitis – contact lens intolerance – anemia, accelerated ESR, increase in transaminase activity – itching, skin inflammation, rash, skin hypersensitivity – muscle and joint pain – abnormal lipid profile
Statins	– pleiotropic effects (anti-inflammatory, antioxidant, antiproliferative and lowers the level of lipids) – inhibition of cell proliferation in the theca layer of the ovaries and reduction of steroid hormone synthesis	– active liver disease (ALT, AST exceeding 3 times the upper limit of normal values) – pregnancy or lactation – hypersensitivity	– muscle damage (myopathy) – increase in liver enzymes in the serum – headache – blurred vision – insomnia – ailments of the digestive tract – rash – joint pain
Fibroblast growth factors (FGFs)	– regulation of carbohydrate and lipid metabolism – cardioprotection – reduction of insulin resistance	– unknown	– unknown
Vitamin D3	– improves insulin sensitivity	– poisoning with or allergy to vitamin D	– myocardial injury – gastrointestinal symptoms: nausea, vomiting, diarrhea – hypercalciuria, polyuria, renal damage – pain in the muscles or joints

Summary

Treatment of polycystic ovary syndrome, a disease affecting a significant part of the population of women worldwide, remains focused on specific targets, which may differ between individual women. The ideal would be causal treatment, but due to the ongoing lack of full understanding of the pathogenesis of the syndrome, is not entirely feasible. However, the increasing amount of research and continually refined new therapeutic options mean that the effects of the available therapies are improving. Despite this, unfortunately, none of them is able to completely eliminate all the symptoms and adverse consequences of PCOS. A comprehensive approach, regular check-ups to prevent remote effects of the disease and a healthy, active lifestyle seem to be the best possible solution.

References

1. Wołczyński S, Zgliczyński W. Abnormalities of the menstrual cycle. In: Large Interna – Endocrinology. 2nd edition. Medical Tribune Poland, Warsaw 2012, 561–567.
2. The Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). *Hum Reprod*. 2004;19(1):41–47. doi: 10.1093/humrep/deh098.
3. Milewicz A. Reimbursement of metformin for polycystic ovary syndrome. *Endokrynol Pol*. 2013;64(5):409–414. doi: 10.5603/EP.2013.0025.
4. Morotti E, Battaglia B, Fabbri R, Paradisi R, Venturoli S, Battaglia C. Cigarette smoking and cardiovascular risk in young women with polycystic ovary syndrome. *Int J Fertil Steril*. 2014;7(4):301–312. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3901187 [Published online 2013 Dec 22].
5. Davies MJ, Marino JL, Willson KJ, March WA, Moore VM. Intergenerational associations of chronic disease and polycistic ovary syndrome. *PLoS One*. 2011;6(10):e25947. doi: 10.1371/journal.pone. 0025947.
6. Tan S, Scherag A, Janssen OE et al. Large effects on body mass index and insulin resistance women with PCOS (FTO) variants in patients with polycystic ovary syndrome (PCOS). *BMC Med Genet*. 2010;11:12. doi: 10.1186/1471-2350-11-12.
7. Fica S, Albu A, Constantin M, Dobri GA, Davila C. Insulin resistance and fertility in polycystic ovary syndrome. *J Med Life*. 2008;1(4):415–422. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3018970/ Published online 2008 Nov 25.
8. Traub ML. Assessing and treating insulin resistance in women with polycystic ovarian syndrome. *World J Diabetes*. 2011;2(3):33–40. doi: 10.4239/wjd.v2.i3.33.
9. Sathyapalan T, Atkin ST. Mediators of inflammation in polycystic ovary syndrome in relation to adiposity. *Mediat Inflamm*. 2010;2010: 758656. doi: 10.1155/2010/758656.
10. McClung J, Karl P. Iron deficiency and obesity: The contribution of inflammation and diminished iron absorption. *Nutr Rev*. 2009;67: 100–104. doi: 10.1111/j.1753-4887.2008.00145.x.
11. Bentley-Lewis R, Selly E, Dunaif A. Ovarian hypertension: Polycystic ovary syndrome. *Endocrinol Metab Clin North Am*. 2011;40:433–449. doi: 10.1016/j.ecl.2011.01.009.
12. Krępuła K, Bidzińska-Speichert B, Lenarcik A, Tworowska-Bardzińska U. Psychiatric disorders related to polycystic ovary syndrome. *Pol J Endocrinol*. 2012;63(6):488–491.
13. Dumesic DA, Lobo RA. Cancer risk and PCOS. *Steroids*. 2013;78(8): 782–785. doi: 10.1016/j.steroids.2013.04.004.

14. Stovall DW, Bailey AP, Pastore LM. Assessment of insulin resistance and impaired glucose tolerance in lean women with polycystic ovary syndrome. *J Womens Health*. 2011;20(1):37–43. doi: 10.1089/jwh.2010.2053.
15. Sidhwani S, Scoccia B, Sunghay A, Stephens-Archer CN, Mazzone T, Sam S. PCOS is associated with atherogenic changes in lipoprotein particle number and size independent of body weight. *Clin Endocrinol*. 2011;75:76–82. doi: 10.1111/j.1365-2265.2011.04015.x.
16. Bu Z, Kuok K, Meng J, Wang R, Xu B, Zhang H. The relationship between polycystic ovary syndrome, glucose tolerance status and serum preptin level. *Reprod Biol Endocrine*. 2012;10:10. doi: 10.1186/1477-7827-10-10.
17. Domecq JP, Prutsky G, Mullan RJ. Lifestyle modification programs in polycystic ovary syndrome: Systematic review and meta-analysis. *J Clin Endocrinol Metab*. 2013;98(12):4655–4663. doi: 10.1210/jc.2013-2385.
18. Kahal H, Atkin SL, Sathyapalan T. Pharmacological treatment of obesity in patients with polycystic ovary syndrome. *J Obes*. 2011: 402052. doi: 10.1155/2011/402052.
19. Beck E, Scheen AJ. Metformin, an antidiabetic molecule with anticancer properties. *Rev Med Liege*. 2013;68(9):444–449.
20. Chung HH, Moon JS, Yoon JS, Lee HW, Won KC. The relationship between metformin and cancer in patients with type 2 diabetes. *Diabetes Metab J*. 2013;37(2):125–131. doi: 10.4093/dmj.2013. 37.2.125.
21 Badr D, Kurban M, Abbas O. Metformin in dermatology: An overview. *J Eur Acad Dermatol Venereol*. 2013;Nov, 27:1329–1335. doi: 10.1111/jdv.12116.
22. Tang T, Glanville J, Hayden CJ, White D, Barth JH, Balen AH. Combined lifestyle modification and metformin in obese patients with polycystic ovary syndrome: A randomized, placebo-controlled, double-blind multicentre study. *Hum Reprod*. 2006;21:80–89. doi: 10.1093/humrep/dei311.
23. Oppelt PG, Mueller A, Jentsch K, et al. The effect of metformin treatment for 2 years without caloric restriction on endocrine and metabolic parameters in women with polycystic ovary syndrome. *Exp Clin Endocrinol Diabetes*. 2010;118:633–637. doi: 10.1055/s-0029-1237705.
24. Malik SM, Traub M. Defining the role of bariatric surgery in polycystic ovarian syndrome patients. *World J Diabetes*. 2012;3(4):71–79. doi: 10.4239/wjd.v3.i4.71.
25. Wolf JE, Shander D, Huber F, et al. Randomized, double-blind clinical evaluation of the efficacy and safety of topical eflornithine HCl 13.9% cream in the treatment of women with facial hair. *Int J Dermatol*. 2007;46:94–99. doi: 10.1111/j.1365-4632.2006.03079.x.
26. Cakir GA, Erdogan FG, Gurler A. Isotretinoin treatment in nodulocystic acne with and without polycystic ovary syndrome: Efficacy and determinants of relapse. *Int J Dermatol*. 2013;Mar;52(3):371–376. doi: 10.1111/j.1365-4632.2012.05691.x.
27. Çetinözman F, Yazgan AD, Elçin G, Yildiz BO. Insulin sensitivity, androgens and isotretinoin therapy in women with severe acne. *J Dermatolog Treat*. 2014;25(2):119–122. doi: 10.3109/09546634.2012.751482.
28. Vexiau P, Chaspoux C, Boudou P, et al. Effects of minoxidil 2% vs. cyproterone acetate treatment on female androgenetic alopecia: A controlled, 12-month randomized trial. *Br J Dermatol*. 2002, 146(6):992–999. doi: 10.1046/j.1365-2133.2002.04798.x.
29. Vinaixa M, Rodriguez MA, Samino S et al. Metabolomics reveals reduction of metabolic oxidation in women with polycystic ovary syndrome after pioglitazone-flutamide-metformin polytherapy. *PLoS One*. 2011;6(12):e29052. doi: 10.1371/journal.pone.0029052.
31. Du Q, Yang S, Wang YJ, Wu B, Zhao YY, Fan B. Effects of thiazolidinediones on polycystic ovary syndrome: A meta-analysis of randomized placebo-controlled trials. *Adv Ther*. 2012;29(9):763–774. doi: 10.1007/s12325-012-0044-6.
31. Jensterle Sever M, Kocjan T, Pfeifer M, Kravos NA, Janez A. Short-term combined treatment with liraglutide and metformin leads to significant weight loss in obese women with polycystic ovary syndrome and previous poor response to metformin. *Eur J Endocrinol*. 2014;170(3):451–459. doi: 10.1530/EJE-13-0797.
32. Thomson RL, Spedding S, Brinkworth GD, Noakes M, Buckley JD. Seasonal effects on vitamin D status influence outcomes of lifestyle intervention in overweight and obese women with polycystic ovary syndrome. *Fertil Steril*. 2013;99(6):1779–1785. doi: 10.1016/j.fertnstert.2012.12.042.
33. Celik O, Acbay O. Effects of metformin plus rosuvastatin on hyperandrogenism in polycystic ovary syndrome patients with hyperlipidemia and impaired glucose tolerance. *J Endocrinol Invest*. 2012;35(10):905–910. doi: 10.3275/8371.
34. Sathyapalan T, Shepherd J, Coady AM, Kilpatrick ES, Atkin SL. Atorvastatin reduces malondialdehyde concentrations in patients with polycystic ovary syndrome. *J Clin Endocrinol Metab*. 2012;97(11): 3951–3955. doi: 10.1210/jc.2012-2279.
35. Kimura Y, Hyogo H, Yamagishi S, et al. Atorvastatin decreases serum levels of advanced glycation endproducts (AGEs) in nonalcoholic steatohepatitis (NASH) patients with dyslipidemia: Clinical usefulness of AGEs as a biomarker for the attenuation of NASH. *J Gastroenterol*. 2010;45(7):750–757. doi: 10.1007/s00535-010-0203-y.
36. Macut D, Bjekić-Macut J, Savić-Radojević A. Dyslipidemia and oxidative stress in PCOS. *Front Horm Res*. 2013;40:51–63. doi: 10.1159/000341683.
37. Salehpour S, Tohidi M, Akhound MR, Amirzargar N. N-acetylcysteine: A novel remedy for polycystic ovarian syndrome. *Int J Fertil Steril*. 2009,3:66–73. https://www.researchgate.net/publication/228674382_N_Acetyl_Cysteine_A_novel_Remedy_for_Poly_Cystic_Ovarian_Syndrome. Accessed 23 Aug 2009.
38. Lansdown A, Rees DA. The sympathetic nervous system in polycystic ovary syndrome: A novel therapeutic target? *Clin Endocrinol (Oxf)*. 2012;77(6):791–801. doi: 10.1111/cen.12003.
39. Schlaich MP, Straznicky N, Grima M, et al. Renal denervation: A potential new treatment modality for polycystic ovary syndrome? *J Hypertens*. 2011;29(5):991–996. doi: 10.1097/HJH.0b013e328344db3a.
40. Katoh M. FGFR2 abnormalities underlie a spectrum of bone, skin, and cancer pathologies. *J Invest Dermatol*. 2009;129:1861–1867. doi: 10.1038/jid.2009.97.
41. Kharitonenkov A, Adams AC. Inventing new medicines: The FGF21 story. *Mol Metab*. 2014;3:221–229. doi: 10.1016/j.molmet.2013.12.003.
42. Unfer V, Carlomagno G, Dante G, et al. Effects of myo-inositol in women with PCOS: A systematic review of randomized controlled trials. *Gynecol Endocrinol*. 2012;28(7):509–515. doi: 10.3109/09513590.2011.650660.
43. Erin Barthelmess Rajesh KN. Polycystic ovary syndrome: Current status and future perspective. *Front Biosci*. 2014;6:104–119. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4341818/. Published online 2014 Jan 1.
44. Sunita J. Ramanand, Balasaheb B. Ghongane, Jaiprakash B. Ramanand, et al. Clinical characteristics of polycystic ovary syndrome in Indian women. *Indian J Endocrinol Metab*. 2013;17(1):138–145. doi: 10.4103/2230-8210.107858.

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